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CXXVIII. DETERMINATION OF THE ANTI-HAEMORRHAGIC VITAMIN

BY H. J. ALMQUIST AND A. A. KLOSE

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(Received 12 April 1939)

IN an earlier paper [Almquist *et al.* 1938] we presented a procedure for vitamin K assay. This method has been improved and is now capable of very satisfactory accuracy. We wish to give here the details of our improved method. For the convenience of those interested in using this method we have repeated some details already reported.

METHODS

Single-comb White Leghorn chicks are placed at hatching in metal, wire floored, electrically heated battery brooders. For the first week they are fed only the basal ration which consists of:

Sardine meal, ether-extracted	17.5
Dried brewer's yeast,* ether-extracted	7.5
Salt†	1.0
Cod liver oil	1.0
Calcium carbonate	0.5
Ground polished rice	72.5
					100.0

* Obtained from Northwestern Yeast Co., Chicago, Illinois.

† Contains 0.5% manganese in the form of manganous sulphate.

The diet contains approximately 18.5% protein, 1.1% Ca and 0.5% P. It permits satisfactory growth and freedom from nutritional disturbances other than vitamin K deficiency, which becomes severe within 7–12 days. The level of yeast used in the diet does not become a limiting factor even when lowered much without danger of other nutritional deficiencies.

Maintenance on the basal diet for 1 week aids in eliminating weak or defective animals and reduces the vitamin K reserves of the chicks before the start of the actual assay.

Supplements of vitamin K are included in the diets by mixing them in thoroughly either as a dilute solution or as a fine powder. The chicks are maintained on these diets for 2 weeks, i.e. until 3 weeks old. As shown in a former paper [Almquist *et al.* 1938], chicks do not attain a close adjustment of average blood clotting times to the vitamin K level of the diet until about 3 weeks of age. Attempts to shorten the assay time have not been given more variable and less satisfactory results.

For the determination of the clotting ability of the blood we have now adapted to vitamin K assay the "prothrombin time" method of Quick [1937]. Blood is rapidly and conveniently obtained by cutting off the head of the chick with scissors. The bird may be held in the left hand with feet between thumb and forefinger in such a way as to direct the blood into a tube calibrated to 2 ml. and containing 0.2 ml. of 0.1 M sodium oxalate solution. When blood has been drawn to the 2 ml. mark, the tube is thoroughly shaken. Individual oxalated

blood samples are obtained in this way from all chicks in a group. Slight contamination of the blood with tissue juice is of little importance in this method, since an excess of tissue juice is added to the sample later. This convenience may be contrasted with the extreme precaution against admixture with tissue juice necessary in the assay method given by Dam & Glavind [1938].

For a clotting agent, an extract of chicken breast muscle is prepared [Dam & Glavind, 1938]. Approximately 10 g. of breast muscle from a chicken killed by bleeding are ground with sand and 10 ml. 0.85 % NaCl solution. The mixture is centrifuged and filtered on coarse paper. The resulting liquid is stored in a refrigerator. It is preferable to make a fresh tissue extract for each group of tests. When used, the extract is diluted with the salt solution to a concentration representing 5 % of muscle. This concentration has proved ample in our experiments. The diluted clotting agent (thrombokinas) at a concentration of 5 % is then mixed with an equal volume of 0.025 *M* CaCl₂ solution. The mixture should clot the blood of a normal chicken in 25 to 30 sec. when tested as in the present procedure.

Samples of 0.1 ml. of the oxalated blood are pipetted into small cylindrical vials, 15 × 50 mm., with a flat bottom. A 0.2 ml. portion of the mixed thrombokinas-CaCl₂ solution is run into a vial and a stop-watch started simultaneously. The vial is placed at once in a thermostat adjusted to 38.5–39.0°. A device is employed to tilt the vial to an angle of approximately 45° and back to a vertical position once per second. The end-point, i.e. clot formation, occurs very sharply. The watch is stopped when the bottom of the vial remains covered by a definite gelatinous film. The procedure is repeated to obtain a check measurement. Almost invariably the duplicate time measurements agree very closely, often to one second.

In this work, blood clotting times were also obtained by our former method of puncturing a wing vein, previous to taking the larger sample of blood for prothrombin time measurements. This was done to permit further study of the linear relation between the reciprocal of the blood clotting time and the logarithm of the vitamin K level in the diet [Almquist *et al.* 1938], with a view to employing this relation in an accurate assay procedure. In some cases the prothrombin times of plasma were also obtained after measurements of the whole blood prothrombin time by centrifuging the remaining oxalated blood and repeating the procedure on the clear plasma. This was done to furnish a basis for deciding whether to employ whole blood or plasma in later work. Finally, we also wished to decide whether the reciprocal prothrombin times bear the same relation to the logarithm of the dietary vitamin K level that is shown by the simple clotting times.

The source of vitamin K used in this work was our reference standard solution, 1 ml. of which represents 1 g. of dried alfalfa.

RESULTS AND DISCUSSION

In Table I are given results of our first comparative study of blood clotting times and whole blood prothrombin times as related to the relative vitamin K level in the ration. The chicks, fed according to the usual schedule, received the supplemented diets from the end of the first to the end of the third week, at which time the measurements were made.

It was apparent that the whole blood prothrombin times were subject to far less variability than were the simple blood clotting times, which led us to believe that the former is a more consistent measurement of the prothrombin content of

Table I. Means, standard errors and coefficients of variability of simple blood clotting times and of whole blood prothrombin times. Exp. A

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Blood clotting time		Prothrombin time	
		Mean and standard error (min.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability
4	10	7.97 \pm 1.78	66.9	43.6 \pm 3.5	24.4
8	9	4.38 \pm 0.23	15.1	33.6 \pm 1.4	11.6
10	11	3.50 \pm 0.86	73.4	31.2 \pm 2.1	20.0
16	10	2.63 \pm 0.55	65.8	30.1 \pm 0.5	5.2

blood owing, probably, to effective elimination of thrombokinase and Ca ion variations. The speed and reproducibility of the prothrombin time measurements appeared as further advantages.

The relation of reciprocal simple clotting time to the logarithm of the relative vitamin K dietary level (Fig. 1, curve A) showed the same linear character as reported formerly. Similarly, the reciprocal average prothrombin times exhibited

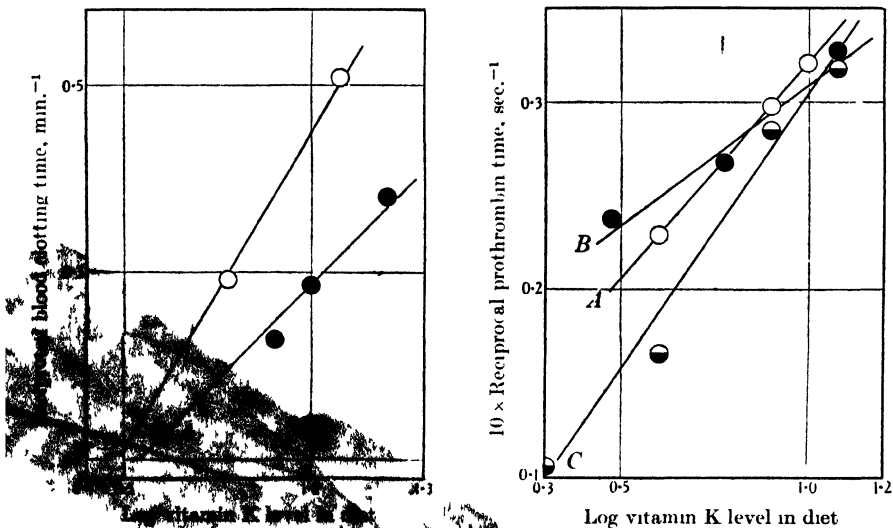


Fig. 2.

a closely linear relation to the dietary vitamin K level for the three lower level (Fig. 2, curve A) but dropped below the expected value in the case of the highest vitamin K level. The results suggested that a maximum level of 10 or 12 ml./kg. of diet should not be exceeded if the linear relation is expected to hold.

In a second experiment we obtained individual prothrombin time measurements on the plasma also. Results are given in Table II.

The same consistent relation between reciprocal times and the logarithms of the vitamin K levels is shown by this experiment (Fig. 1, curve B and Fig. 2, curve B). As in the first experiment, the variability of the prothrombin times was much less than that of the simple clotting times.

The mean plasma prothrombin time was greater than that for whole blood. It was noted in this and in subsequent studies that centrifuging caused a small and very irregular prolongation of the prothrombin time. In very deficient

Table II. *Means, standard errors and coefficients of variability of simple blood clotting times, whole blood prothrombin times and plasma prothrombin times. Exp. B*

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Blood clotting time		Whole blood prothrombin time		Plasma prothrombin time	
		Mean and standard error (min.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability	Mean and standard error (sec.)	Coefficient of variability
3	15	10.07 ± 2.31	61.1	42.0 ± 1.9	17.1	51.2 ± 1.8	9.1
6	15	3.42 ± 0.55	60.2	37.3 ± 1.0	9.6	40.2 ± 1.0	9.3
12	15	1.97 ± 0.37	72.1	30.5 ± 0.5	6.1	—	—
5*	14	3.71 ± 0.64	62.2	40.6 ± 2.9	25.9	49.3 ± 3.0	21.7

* A separate group included here for additional comparison.

blood the prothrombin time of plasma approached that of the whole blood. It appears that centrifuging the blood added no further accuracy to the method and perhaps caused, in cases of short prothrombin times, a distinct, variable loss of prothrombin (or thrombin). Examples illustrating these statements are given in Table III.

Table III. *Comparison of individual whole blood and plasma prothrombin times from the same blood samples*

Whole blood prothrombin time sec.	Plasma prothrombin time sec.
25	27
29	37
30	41
31	37
40	50
40	37
40	47
40	49
47	50
50	54
59	64
67	72
76	79
235	267
315	343
355	378

Notwithstanding the irregular differences introduced by centrifuging, it was found in several experiments that the correlation between individual whole blood prothrombin time and plasma prothrombin time was high, coefficients of 0.813, 0.975 and 0.930 being obtained. It was decided to continue with the use of whole blood rather than plasma since the former seemed to yield very consistent results and to offer a simpler procedure through the elimination of centrifuging. An additional advantage is that the formation of a clot is much more easily seen in whole blood than in the nearly colourless plasma.

In a further experiment we attempted to get longer whole blood prothrombin times in at least one group in order to extend the lower range of the observations. Results obtained in this experiment are given in Table IV.

The relation of the reciprocal mean prothrombin time to the logarithm of the vitamin K dietary level is given by curve *C* of Fig. 2. The results of this experiment are a little more variable than might have been expected, owing to the fact

Table IV. *Means, standard errors and coefficients of variability of whole blood prothrombin times in relation to relative vitamin K level in the diet. Exp. C.*

Ref. standard of vitamin K per kg. diet ml.	No. of chicks	Mean prothrombin time (sec.)	Standard error	Coefficient of variability
2	16	94.4	± 6.8	27.8
4	16	60.2	± 3.4	21.9
8	12	35.1	± 2.8	26.1
12	16	31.3	± 1.1	13.0

that blood samples were taken at 2½ weeks rather than at 3 weeks. It is obvious, however, that the same linear relation holds as noted in former cases.

By the use of this line we were able to evaluate in terms of the reference standard one of our preparations of the molecular compound of vitamin K with deoxycholic acid [Almquist & Klose, 1939]. This preparation was fed at levels of 25 and 50 mg./kg. diet to other chicks of the same lot as used in Exp. C and over the same interval of time. Average prothrombin times obtained were, respectively, 83 and 51 sec. By graphical interpolation we obtained equivalent volumes of the reference standard solution of 2.34 and 4.47 ml. respectively. The activity of this preparation is, therefore, 0.094 ml. of reference standard per mg. in one determination and 0.089 ml./mg. in the other, values which are in very fair agreement. In each series of assays it is necessary to include at least two groups of chicks treated with the reference standard solution at levels, usually, of 2 ml. and 10 ml./kg. of diet, in order to establish the line for interpolating the activities of supplements administered simultaneously. This procedure is now being employed by us with very satisfactory results.

On several occasions calculations were made of the coefficients of correlation between individual simple blood clotting times and prothrombin times within groups of chicks. Values obtained were 0.651, 0.363 and 0.610, showing definite but not high correlation. On the other hand, the correlation between the mean simple blood clotting times and the mean prothrombin times in a series of groups receiving different amounts of vitamin K was found to be expressed by the coefficient 0.89 in one case and 0.99 in another, indicating that the two mean times may serve with approximately equal accuracy when groups of sufficient size are used. The much lower variability of the prothrombin times together with the fact that conveniently measurable values for prothrombin time can almost invariably be obtained, even when simple clotting times are greatly prolonged, has led us to adopt the prothrombin time measurement in our assay procedure. We occasionally employ the simple clotting times when it is desired to obtain an advance indication of the effect of a vitamin K supplement.

It may be remarked that the range of clotting times within which our assay procedure is conducted is almost entirely below the clotting time of 10 min. which some workers have regarded as "normal" [Thayer *et al.* 1938]. It is obvious that there is a vast difference in the amount of vitamin K required to maintain a clotting time of 10 min. as compared with one of 2 min., for example. Preparations of vitamin K which are stated to produce "normal" clotting time can be compared only on the basis of the relation we have demonstrated.

In a former paper we presented individual blood clotting times of chicks reared upon the same diet. These times showed a large range of values which were fairly constant for an individual. We have since obtained further data showing that chicks of the same age and size and on the same diet can exhibit large consistent differences in simple blood clotting time. It is almost impossible

to account for the consistent individual values by assuming, in the case of the prolonged clotting times, a uniform lack of contamination with tissue juice or, in the case of the short clotting times, a uniform degree of such contamination. The causes of the large variability in simple blood clotting times undoubtedly lie within the blood itself, and it is evident that the prothrombin time measurement disposes of these to a large extent. It is also evident that simple blood clotting times obtained from a few chicks tested only a few hours or days after receiving a vitamin K supplement are likely to be extremely misleading.

SUMMARY

An improved method for conducting a vitamin K assay is described.

The reciprocal of the mean blood clotting time and of the mean prothrombin time bears a linear relation to the logarithm of the vitamin K level in the diet over a practical range of levels.

The authors wish to acknowledge valuable assistance received from Dr I. M. Lerner in the analysis of data.

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CXXIX. FLUORIDE SENSITIVITY OF *PROPIONIBACTERIUM PENTOSACEUM* AS A FUNCTION OF GROWTH CONDITIONS

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(Received 5 May 1939)

THE isolation by Werkman *et al.* [1936] of phosphoglyceric acid from a bacterial fermentation of glucose suggested the occurrence of a dissimilation similar to that already proposed for muscle by Embden *et al.* [1933], and extended to yeast by Meyerhof & Kiessling [1934]. The formation of phosphoglyceric acid by the propionic acid bacteria as well as by many other species has been shown by Stone & Werkman [1936, 1, 2; 1937].

That the fermentation of glucose necessarily passes through the intermediate stage of phosphoglyceric acid was questioned by Werkman *et al.* [1937] when proliferating cells were found to dissimilate glucose at a concentration of NaF which inhibited the breakdown of phosphoglyceric acid. It seemed unlikely, therefore, that the dissimilation was proceeding exclusively through this ester. Werkman *et al.* [1937] found that phosphoglyceric acid was readily dissimilated. This fact was rather striking since a satisfactory dissimilation of phosphoglyceric acid by resting cells had not been previously obtained in this laboratory. Inasmuch as the work of Werkman *et al.* [1937] was carried out with proliferating cells, it appeared that significant differences existed between cells grown on phosphoglyceric acid and those obtained from glucose media. Therefore the effect of culturing the organism in the presence of phosphoglyceric acid on its ability to dissimilate this compound was determined. Furthermore, since the organism appeared able to adapt itself, it was of interest to determine whether some similar concept might explain growth in NaF, i.e. whether the organism is adaptive with respect to the path by which glucose is dissimilated. If this suggestion were true organisms grown in phosphoglyceric acid media might acquire the ability to ferment this compound and then dissimilate glucose through this ester as an intermediate. On the other hand, organisms grown in the presence of NaF might lose the ability to ferment phosphoglyceric acid and develop a mechanism dissimilating glucose through some intermediate other than phosphoglyceric acid. Since the breakdown of phosphoglyceric acid is inhibited by NaF, organisms with a "phosphoglyceric acid" glucolytic system should be sensitive to NaF, while those using some other course should be insensitive to this compound.

We have succeeded in obtaining two types of organisms differing in their sensitivity toward NaF and in their ability to ferment phosphoglyceric acid. The evidence supports the suggestion that the difference in behaviour is enzymic.

METHODS

The basal medium was 0.5% yeast extract (Difco) and 1.0 or 1.5% NaHCO_3 buffer. (1) The yeast extract and glucose, (2) NaF and NaHCO_3 and (3) Na phosphoglycerate¹ were sterilized separately by autoclaving. The NaHCO_3 , containing bromothymol blue indicator, was saturated with CO_2 before the other ingredients were added and the flask inoculated from a glucose broth culture of *Propionibacterium pentosaceum* (49 W). Flasks were incubated at 30°; CO_2 was bubbled through the medium during the entire 6.5 day incubation period. The cells were centrifuged, resuspended in 25 volumes of distilled water, allowed to stand 15 min. and again centrifuged, usually for 1 hr. or more. After decanting the wash water, the cells were resuspended in 25 volumes of 0.05 M K phosphate buffer (pH 6.3). The suspensions were tested under N_2 for ability to ferment phosphoglyceric acid and for NaF sensitivity by measuring the CO_2 produced in Barcroft-Warburg manometers.

EXPERIMENTAL

Typical data are presented in Table I. Cells were grown in three flasks, the first containing in addition to the basal medium, 0.8% Na phosphoglycerate and 0.1% glucose, the second 0.02 M NaF and 0.5% glucose and the third 0.5% glucose only. The cells grown in the presence of phosphoglyceric acid were quite active in dissimilating this ester (cup 1), and behaved as though phosphoglyceric acid were an intermediate inasmuch as the fermentation of glucose was entirely inhibited by 0.02 M NaF (cup 2).

Cells grown in NaF and glucose, however, showed a different behaviour. The dissimilation of phosphoglyceric acid was negligible (cup 7), while the gas production from glucose in the presence of NaF (cup 8) was almost as great as in its absence (cup 9). In the majority of our experiments these NaF organisms actually formed more CO_2 in the presence of NaF than in its absence. We shall return to this point. Organisms grown in NaF behaved as organisms using an intermediate mechanism not sensitive to this poison.

It is interesting that the organisms grown in glucose without NaF were quite similar to those grown in phosphoglyceric acid. They dissimilated phosphoglyceric acid readily (cup 12) and were fluoride-sensitive with respect to glucose dissimilation (cups 13 and 14). It might appear that the original supposition that the organisms must be grown on phosphoglyceric acid in order to dissimilate the ester was not true, for the organisms grown on glucose were quite similar to those grown on phosphoglyceric acid. However, an experiment is described later which correlates the fermentation of phosphoglyceric acid and growth on this ester. The observation that phosphoglyceric acid-fermenting, NaF-sensitive organisms can be obtained from glucose simplified further experimentation and obviated the use of phosphoglyceric acid in growing such organisms.

The results described suggest that *Propionibacterium* can dissimilate glucose by at least two paths. By one path, probably involving phosphoglyceric acid, the organism is NaF-sensitive, whereas by the other path (or paths) it is not sensitive. Presumably the latter route does not involve phosphoglyceric acid. It appears that organisms of either type may be obtained by introducing or omitting the poison in the medium.

¹ Kindly provided by Prof. Carl Neuberg as barium phosphoglycerate.

Table I. *Dissimilation of glucose by cells grown in the presence and absence of NaF*

Basal medium plus ...	Cells grown in glucose 0.1%, phosphoglyceric acid 0.8%				
Cup no. ...	1	2	4	5	6
Main chamber	1.7 ml. of organisms in each cup (under nitrogen)				
Side cup	0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	0.3 ml. H ₂ O	0.1 ml. NaF, 0.2 ml. H ₂ O
μ l. CO ₂ evolved					
120 min.	65.7	3.5	96.2	1.5	6.1
310 min.	143.0	2.3	128.7	3.0	6.1
490 min.	152.0	2.3	140.0	1.5	6.1
490 min. with control subtracted	150.5	-3.8	138.5	—	—
Basal medium plus ...	Cells grown in glucose 0.5% NaF 0.02 M				
Cup no. ...	7	8	9	10	11
Main chamber	1.7 ml. of organisms in each cup (under nitrogen)				
Side cup	0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	0.3 ml. H ₂ O	0.1 ml. NaF, 0.2 ml. H ₂ O
μ l. CO ₂ evolved					
120 min.	9.1	83.9	94.9	12.8	14.5
310 min.	22.1	129.7	143.0	19.9	31.9
490 min.	31.2	141.1	156.8	24.2	37.7
490 min. with control subtracted	7.0	103.4	132.6	—	—
Basal medium plus ...	Cells grown in glucose 0.5%				
Cup no. ...	12	13	14	15	16
Main chamber	1.7 ml. of organisms in each cup (under nitrogen)				
Side cup	0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	0.3 ml. H ₂ O	0.1 ml. NaF, 0.2 ml. H ₂ O
μ l. CO ₂ evolved					
120 min.	52.5	22.9	120.0	-2.8	+13.4
310 min.	140.1	31.5	177.9	-11.3	11.9
490 min.	172.2	37.2	190.3	-14.3	13.4
490 min. with control subtracted	184.5	23.8	204.6	—	—

Pga = phosphoglyceric acid 0.25% (0.135 M) (86.5 mg. BaPga + 40 mg. Na₂SO₄ + 1.8 ml. H₂O, filter; make up to 2 ml.).

G (glucose) = 0.125% (0.069 M).

NaF = 0.4 M.

Organisms ~ 4% by volume in 0.05 M phosphate buffer (pH 6.3).

In Table II the cells were harvested after 4 days' growth instead of the usual 6.5 days. One condition, in addition to time, was unavoidably different in this experiment and for this reason it is difficult to say that the somewhat different results are due entirely to a shorter growth period. The yeast extract used was in both cases "Difco," but that used in Exp. I was considerably lighter in colour, indicating differences in composition. The experiment is described here because it shows that the ability of micro-organisms to dissimilate phosphoglyceric acid is affected by growing them in the presence of the acid. The dissimilation of phosphoglyceric acid (cup 1) is rapid when compared with that of organisms grown on NaF and glucose, and glucose (cups 7 and 13 respectively). The greater fermentation of phosphoglyceric acid by "fluoride" organisms than by "glucose" organisms is not in agreement with expectation. In this connexion, it is interesting that the total CO₂ formed is about the same (cups 7 and 13) and the difference becomes apparent when the controls (cups 12 and 18) are subtracted. The NaF sensitivity of cells grown on phosphoglyceric acid does not

Table II. *Dissimilation by cells grown in various media*

Basal medium plus ...			Cells grown in glucose 0.1 %, phosphoglyceric acid 0.8 %					
Cup no.	1	2	3	NaF in- hibition %	5	6
Main chamber			1.7 ml. of organisms in each cup					
Side cup			0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	—	0.2 ml. H ₂ O, 0.1 ml. NaF	0.3 ml. H ₂ O
μ l. CO ₂ * evolved								
30 min.			25.8	12.4	40.6	69.5	1.5	- 1.2
60 min.			34.1	25.0	71.5	72.0	2.9	- 1.2
105 min.			50.3	44.4	100.7	52.6	4.4	+ 0.6
195 min.			83.8	76.8	124.7	38.5	5.8	+ 1.2
330 min.			134	114.8	135.7	15.4	13.1	+ 8.5
765 min.			170	145.5	151.7	4.1†	16.1	+ 12.2
765 min. with control subtracted			157.8	—	—	—	—	—
Basal medium plus ...			Cells grown in glucose 0.5 %, NaF 0.02 M					
Cup no.	7	8	9	NaF in- hibition %	11	12
Main chamber			1.7 ml. of organism in each cup					
Side cup			0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	—	0.2 ml. H ₂ O, 0.1 ml. NaF	0.3 ml. H O
μ l. CO ₂ * evolved								
30 min.			16.9	38.3	30.1	- 27.5	4.3	1.5
60 min.			18.2	72.0	59.0	- 22.0	8.7	2.9
105 min.			23.4	122.1	104.2	17.1	14.5	5.8
195 min.			32.4	133.6	123.2	8.4	24.6	8.8
330 min.			55.8	173.6	140.5	- 73.6	40.6	19.0
765 min.			72.0	197.7	151.2	- 30.7	50.8	24.8
765 min. with control subtracted			47.2	—	—	—	—	—
Basal medium plus ...			Cells grown in glucose 0.5 %					
Cup no.	13	14	15	NaF in- hibition %	17	18
Main chamber			1.7 ml. of organisms in each cup					
Side cup			0.2 ml. Pga, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	—	0.2 ml. H ₂ O, 0.1 ml. NaF	0.3 ml. H ₂ O
μ l. CO ₂ * evolved								
30 min.			11.5	11.1	25.9	57.1	11.0	8.3
60 min.			15.8	24.9	60.3	58.7	16.5	13.9
105 min.			21.5	48.2	87.3	44.8	22.1	16.6
195 min.			31.5	81.4	127.2	36.0	37.2	18.0
330 min.			50.1	112.7	127.8	11.8	52.4	45.9
765 min.			65.9	122.8	143.6	14.5	68.9	51.4
765 min. with control subtracted			14.5	-	—	—	—	—

Solutions and organism as in Table I.

* Controls have been subtracted in cups 2, 3, 8, 9, 14 and 15.

† The comparison after a few hr. is not quantitatively accurate, because the substrate concentration is no longer the same.

differ greatly from that of cells grown on glucose, but culturing in phosphoglyceric acid does have some effect, the cells being about 14% more sensitive.

Neither the "phosphoglyceric acid" nor "glucose" cells of this experiment are as sensitive to NaF as are those of Exp. 1. Since the growing time was less, there is the possibility that as the growing time increases a NaF-insensitive system is replaced by a NaF-sensitive one and that in the case in question this change has not yet been completed.

The point is that organisms may more rapidly acquire the ability to break down phosphoglyceric acid when grown on this ester than on glucose.

Complete inhibition of dissimilation

By measuring the anaerobic CO₂ production of *P. pentosaceum* only a small part of the total products is determined. It seemed possible that the variation observed between the two types of organisms might lie in the CO₂ formation and not apply to the other products common to the propionic fermentation.

Since the products of the propionic acid fermentation are entirely acids and CO₂, this point can be tested, using the respirometer, by suspending the organisms in NaHCO₃ buffer. The technique was that used by Fromageot & Chaix [1937].

The two types of organisms, grown 6.5 days on glucose and on glucose plus NaF, were washed with distilled water as previously and suspended in a buffer containing 0.20 % NaHCO₃ and 0.9 % NaCl. Substances to be added were dissolved in the same buffer. After saturation with CO₂ which had been passed over hot copper gauze, the suspension and solutions were placed in the manometer cups. The air in the manometer cups was then displaced by O₂-free CO₂.

The results (Table III) show that NaF entirely inhibited both acid and CO₂ formation by organisms grown in glucose. On the other hand, acid and CO₂

Table III. *NaF sensitivity of Propionibacterium pentosaceum as a function of growth conditions*

Basal medium plus ...		Cells grown in glucose 0.5 %				
Cup no.	...	1	2	3	4	5
Main chamber		1.7 ml. of organisms in each cup				
Side cup		0.4 ml. Pga	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. buffer	0.2 ml. buffer, 0.1 ml. NaF	0.3 ml. buffer
μ l. CO ₂ * evolved						
30 min.		- 4.8	+ 14.1	112.6	8.6	1.4
80 min.		8.9	22.4	452.9	15.7	+ 1.3
230 min.		+ 27.8	+ 18.4	548.3	17.8	- 6.4
470 min.		116.6	+ 38.0	575.8	36.4	+ 15.4
950 min.		229	+ 56	626.7	51.4	+ 33.8
950 min. with control subtracted		195	+ 4.6	592.9	—	—
Basal medium plus ...		Cells grown in glucose 0.5 %, NaF 0.02 M				
Cup no.	...	6	7	8	9	10
Main chamber		1.7 ml. of organisms in each cup				
Side cup		0.4 ml. Pga	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. buffer	0.2 ml. buffer, 0.1 ml. NaF	0.3 ml. buffer
μ l. CO ₂ * evolved						
30 min.		+ 10.4	38.4	106.8	6.9	- 0.2
80 min.		+ 12.5	247.5	417.9	12.3	+ 2.5
230 min.		+ 21.3	593.3	453.3	13.7	- 2.7
470 min.		35.1	673.1	484.4	32.9	+ 15.7
950 min.		62.6	733.6	532.3	46.5	24.0
950 min. with control subtracted		38.6	687.1	508.3	—	—

Pga = 0.125 % in NaHCO₃-NaCl buffer.

G (glucose) + NaF: same as in Table I but made up in NaCl-NaHCO₃ buffer.

* Corrections have been made for small spontaneous evolution of gas from buffer, and increase of CO₂ solubility due to addition of Pga solution to cups 1 and 6. This disturbance probably explains retarded gas evolution in cup 1.

productions by organisms grown in the presence of NaF were only slightly inhibited by the poison in the earlier stages of the dissimilation and in the later stages the CO₂ evolution increased in the presence of NaF to a value which was higher than in its absence. The entire dissimilation is affected by the NaF, and not only the CO₂ production.

Permeability of bacterial cells to NaF

The difference in ability of the two types to ferment phosphoglyceric acid is apparently not due to the presence of NaF inside the cells grown in the poison, as might be the case if the fluoride were not thoroughly washed from the cells.

Dilution of the cells with phosphate buffer reduces the concentration of the NaF to 0.0008 *M*; moreover, washing with distilled water should remove the greater part of the fluoride before the dilution occurs. Furthermore, soaking the cells overnight in distilled water at 12° did not increase the phosphoglyceric fermentation above that of cells washed in the ordinary manner, as would be expected if some poison left in the cells of the "sodium fluoride" organisms was responsible for the small phosphoglyceric fermentation. It therefore appears that the cells are enzymically different. On the other hand, if we assume that the fluoride is not washed from the cells, we are led to the same general conclusion that the cells are enzymically different, for the dissimilation of glucose would then be proceeding in the presence of NaF contrasting with the fluoride-sensitive type.

Table IV. *Permeability experiments*

Basal medium ...	Cells under N ₂ during soaking period					
Cup no. ...	1	2	3	4	5	6
In main chamber with 0.16 ml. of cells during 7.5 hr. soaking period	0.1 ml. H ₂ O	0.1 ml. H ₂ O	0.1 ml. NaF	0.1 ml. H ₂ O	0.1 ml. NaF	0.1 ml. H ₂ O
Side cup contents tipped in after soaking period	0.2 ml. G	0.2 ml. G	0.2 ml. G	0.3 ml. H ₂ O	0.3 ml. H ₂ O	0.1 ml. NaF, 0.2 ml. H ₂ O
μl. CO ₂ evolved						
35 min.	51.6	38.4	43.6	7.1	2.9	6.1
275 min.	200.0	147.0	222.0	31.2	35.0	34.2
780 min.	313.0	255	342	103.0	110.0	102.0
780 min. with control subtracted	211.0	152.0	232.0	---	---	---
Basal medium ...	Cells under air during soaking period; N ₂ before tipping					
Cup no. ...	7	8	9	10	11	12
In main chamber with 0.16 ml. of cells during 7.5 hr. soaking period	0.1 ml. H ₂ O	0.1 ml. H ₂ O	0.1 ml. NaF	0.1 ml. H ₂ O	0.1 ml. NaF	0.1 ml. H ₂ O
Side cup contents tipped in after soaking period	0.2 ml. G, 0.1 ml. NaF	0.2 ml. G, 0.1 ml. H ₂ O	0.2 ml. G, 0.1 ml. H ₂ O	0.3 ml. H ₂ O	0.3 ml. H ₂ O	0.1 ml. NaF, 0.2 ml. H ₂ O
μl. CO ₂ evolved						
35 min.	50.6	34.4	45.4	11.4	11.6	12.7
275 min.	205.0	135.8	223.0	45.5	56.6	52.6
780 min.	323.0	226.0	338.2	126.8	121.8	132.8
780 min. with control subtracted	190.2	99.2	216.4	---	---	---

Solutions and organism as in Table I.

To investigate the possibility that cells grown in the presence of NaF are enzymically the same as those grown in glucose alone but differ only in permeability, experiments similar to those of R nnstr m *et al.* [1937] were carried out. Working with bottom yeast, these investigators found a relationship between respiration and permeability. Under anaerobic conditions fermentation was inhibited by NaF; aerobically, respiration was affected only when the cells were soaked in NaF previous to glucose addition, and then inhibition was not complete. With cells in which the reserve had been depleted, however, a short period of soaking aerobically in NaF before glucose was added produced an almost complete inhibition of respiration. It was therefore suggested that impermeability was associated with respiration, the cells being less permeable when respiration was proceeding, and quite permeable under anaerobic conditions. Since all our experiments were carried out under N_2 or CO_2 , the bacterial cells would be permeable to NaF. However, even if the cells are relatively impermeable, one might expect that on soaking in NaF aerobically and anaerobically for a considerable period before introducing the glucose there should occur some penetration and hence some inhibition.

As is seen in Table IV, however, 7 hr. soaking in NaF both anaerobically and aerobically before introducing the glucose caused no inhibition at all. In fact when the cells were soaked in NaF before glucose was added (cups 3 and 9) the CO_2 evolution was of the same order as when NaF was added with the glucose (cups 1 and 7) and in all of these, evolution was greater when NaF was added (cups 2 and 8). These results indicate that the NaF insensitivity of these cells is enzymic in nature, and not due to a permeability factor.

Stimulating effect of NaF on CO_2 evolution

It is not clear why the presence of NaF should cause an increase in CO_2 evolution. The effect was frequently noticed in the absence of glucose (Table II, cups 5 and 6, 11 and 12, 17 and 18; Table III, cups 4 and 5, 9 and 10) but was not as pronounced as in the presence of glucose (Table II, cups 8 and 9; Table III, cups 1 or 3 and 2, 7 or 9 and 2; Table IV, cups 7 and 8). The increase was not due to the effect of NaF on the solubility of CO_2 inasmuch as the addition of NaF to $NaHCO_3$ -NaCl solution saturated with CO_2 did not measurably increase the small spontaneous evolution of gas.

Needham & Lehmann [1937, 2] mentioned increased autoglycolysis caused by NaF. However, since the effect seems to be greater in the presence of glucose we cannot ascribe it entirely to increased autoglycolysis unless the presence of glucose stimulates the endogenous metabolism. A greater glycolysis with two substrates than the sum of each has been reported by Haarmann [1932] working with vertebrate tissues.

However, NaF produces stimulation, its effects appear to be enzymic in character, and therefore to be additional evidence that NaF penetrates to the enzyme centres.

Purity of the culture

The organisms obtained from glucose and from glucose plus NaF medium were alike to all appearances. Werkman *et al.* [1937] showed that the fermentation occurring in the presence of NaF was a normal propionic fermentation. The proportion of CO_2 liberated was of the order to be expected from the propionic fermentation as was also the production of acid and CO_2 indicated by fermentation in $NaHCO_3$ buffer (Table III). Morphologically and in staining reactions the cells grown in the absence and presence of NaF were alike and characteristic of the species.

DISCUSSION

Evidence for the existence of more than one mechanism of glycolysis in animal tissue is not wanting. Ashford & Holmes [1929] have shown that separate mechanisms exist for glucose and glycogen in brain tissue, and the work of Bumm & Fehrenbach [1931] shows that separate systems attack glucose and glycogen in white muscle. Furthermore, according to these investigators a marked substrate preference occurs among various tissues, and those which act preferentially on glucose are activated by a different coenzyme from that of the systems which act upon glycogen. A non-phosphorylating mechanism has been suggested by Needham & Lehmann [1937, 1] for embryo glucolysis.

Two mechanisms of dissimulation by the propionic acid bacteria were suggested by Wood *et al.* [1937]. Later Werkman *et al.* [1937] showed that the dissimulation of glucose by growing propionic acid bacteria was not inhibited by NaF and suggested therefore that the mechanism not involving phosphoglyceric acid was the more active.

In the present communication it has been shown that the organisms are apparently adaptable in the way in which they dissimilate glucose. These results may explain why an organism produces quantities of phosphoglyceric acid, and yet grows in the presence of NaF, and also the variable results obtained when attempting to isolate the ester.

It should perhaps be emphasized that the suggestion that only one mechanism proceeds through phosphoglyceric acid does not imply that only one route is a phosphorylating one. NaF is not necessarily a specific inhibitor for phosphoglyceric acid. It is logical to assume, however, that in this case the fluoride inhibits phosphoglyceric acid dissimulation, for it has been shown that the organism in question produces this compound.

A remark regarding the significance of phosphoglyceric acid dissimulation is pertinent. A compound which is an intermediary will break down at least as rapidly as the original substrate. In the experiments described above the dissimulation of phosphoglyceric acid by the NaF-sensitive type of organisms is only half as great as that of glucose. However, the function of phosphoglyceric acid as an intermediary according to the Embden-Meyerhof-Parnas scheme is not to arise and then break down by dismutation. Its occurrence by oxidation of triosephosphate is followed by intramolecular rearrangements to phosphopyruvic acid whose dephosphorylation is facilitated by the simultaneous phosphorylation of carbohydrate (through adenylic acid as a phosphate carrier). The pyruvic acid, or in yeast the acetaldehyde arising from it, reacts with the triosephosphate which is constantly being formed. Thus the speed of dissimulation of phosphoglyceric acid cannot be compared with that of glucose.

SUMMARY

It has been shown that two types of cells of *Propionibacterium pentosaceum* differing in NaF sensitivity and ability to ferment phosphoglyceric acid, result from culturing in the presence and absence of NaF.

Evidence suggests that the differences are enzymic and it therefore appears that an organism may develop more than one mechanism of dissimilating glucose, depending upon environmental conditions.

The results obtained may be due to training by which a few organisms grow and multiply more rapidly than the others, thus leading to a culture able to bring about the dissimulation of the substrate in question. On the other hand

adaptation of the specific cells may occur as with galactozymase in yeast [Stephenson & Yudkin, 1936]. The present work was not designed to determine this point.

The phosphoglyceric enzyme system described here is always present; thus it is constitutive in the sense of Virtanen & Karström [Karström, 1936].

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CXXX. THE DETERMINATION OF AMINO-NITROGEN USING A COPPER METHOD

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To determine the degree of hydrolysis of proteins by enzymes, as shown by the production of free α -amino-N, it is convenient to have a method which can be carried out rapidly. The methods of Van Slyke [1911; 1912], Sørensen [1907] or Willstätter & Waldschmidt-Leitz [1921] are generally used, but to each of these some objection has been raised. To obtain consistent results for enzymic digests with Van Slyke's method it is necessary to shake for 20-30 min. The other methods do not give very definite titration end-points. Richardson [1934, 1] has considered the principles underlying the formaldehyde, alcohol and acetone titration methods and has shown that the formaldehyde and alcohol methods measure the amino groups whereas the acetone method of Linderstrøm-Lang [1928] measures the carboxyl groups. In a second paper [1934, 2] he gives a full account of the limitations of the Van Slyke method, but concludes that it is to be preferred for dealing with coloured biological products. Of the various titration methods depending on the physico-chemical properties of complex mixtures none was considered wholly satisfactory.

Kober & Sugiura [1912; 1913] and Kober [1917] suggested a method for determining α -amino-N based on the formation of copper salts. Utkin [1933] further modified the method for determination of amino-N in protein decomposition products. Attempts to use the methods of Kober & Sugiura and Utkin in these Laboratories were not very successful. Pure amino-acids did not give theoretical results, and protein digests behaved variably. The copper salt method appeared to offer many advantages, and after further modification by one of us (C. G. P.) it was found to be possible to obtain correct results with certain amino-acids and repeatable results with tryptic digests of proteins. The procedure has certain limits as with other methods for determining amino-N; although, however, it is not entirely specific for amino-N, non-specific reacting substances are unlikely to be present in enzymic digests of proteins.

The method depends on the formation of soluble copper compounds through the reaction between the amino-acid or digest material and excess copper present in the form of copper phosphate. Kober & Sugiura used freshly prepared copper hydroxide and Utkin added copper chloride to the material under test, followed by sodium hydroxide to precipitate the excess copper. Our own observations showed that many of the difficulties encountered were due to the presence of copper in the form of hydroxide and it was not until we substituted copper phosphate that reliable results were obtained. The copper phosphate prepared as described in this paper gives no blank value due to traces of soluble copper. The amount of copper taken into solution by amino-acids or similar material is determined iodimetrically.

Some amino-acids form sparingly soluble copper salts under these conditions (e.g. cystine, methionine, tryptophan, leucine and phenylalanine) and for these

in pure state the method does not work. When mixed with other amino-acids most of these form mixed copper salts which are soluble, and can therefore be estimated by making mixtures with known amounts of a suitable acid (glycine or aspartic acid¹). The proportion of soluble to insoluble acid should be at least 4 to 1. With enzymic digests, the copper compounds appear to be completely soluble; this is also true for proteins hydrolysed with 20% HCl.

Both proline and hydroxyproline are estimated by the copper method, but not by that of Van Slyke. Ammonia, which interferes in the latter, does not affect the copper estimation. The accuracy of the method is easily determined for pure amino-acids, but can only be determined indirectly for more complex mixtures. Parallel determinations carried out by Van Slyke's method and the copper method have given good agreement on tryptic digests of fibrin provided that ammonia is absent and shaking with nitrous acid is continued for 20–30 min. The usual period of about 4 min. is insufficient with partly degraded proteins. In this paper we give the results of determinations on pure amino-acids showing the accuracy of the method and examples of its application to various forms of protein hydrolysates.

EXPERIMENTAL

Reagents required

Copper chloride. 27.3 g. dissolved in 1 l. of water (0.16 M).

Trisodium phosphate. 64.5 g. of disodium hydrogen phosphate dissolved in 500 ml. of CO₂-free distilled water; add 7.2 g. NaOH and when dissolved make to 1 l.

Borate buffer. 57.21 g. of sodium borate are dissolved in 1500 ml. water, 100 ml. of N HCl added, and the volume made to 2 l.

Copper phosphate suspension. The copper chloride solution (1 vol.) is added to the trisodium phosphate solution (2 vol.) and mixed well; 2 vol. of the borate buffer are added. This suspension appears to keep quite well and enough to last for several days may be prepared.

Thymolphthalein. 0.25 g. in 100 ml. of 50% ethyl alcohol.

Sodium thiosulphate. A stock solution approximately 0.1 N prepared by dissolving 49.6 g. of sodium thiosulphate in 200 ml. of CO₂-free distilled water and diluting to 2000 ml. The addition of 0.1% sodium borate keeps the solution stable and dilutions of 0.01 or 0.005 N are made from this stock using water containing borate.

Standard potassium iodate. 0.35675 g. of "Analar" potassium iodate dried at 110° for 1 hr. dissolved to 1000 ml. Used to standardize the thiosulphate solution.

Starch solution. Some difficulty was experienced in preparing a starch solution suitable for this method, but the following preparation works well. Soluble potato starch (20 g.) is mixed with about 50 ml. distilled water and 2000 ml. of boiling water are added. The solution is heated on a water bath and treated with 200 ml. N NaOH. When clear (after about 30 min.) the solution is cooled and 200 ml. N HCl are added. The reaction is adjusted to pH 7 and the solution shaken with 5 g. charcoal and filtered. The charcoal treatment should be repeated if necessary until the filtrate is water clear, after which solid NaCl is added to saturation.

¹ Under the conditions of these experiments aspartic acid forms a soluble copper complex different from the sparingly soluble copper salt commonly employed in the isolation of this amino-acid.

Method

The protein hydrolysate or amino-acid solution is measured into a 50 ml. volumetric flask; 4 drops of thymolphthalein are added, followed by *N* NaOH to a faint blue colour. Then 30 ml. of the copper phosphate suspension are added (measured quite roughly) and the volume made to 50 ml. with distilled water, well mixed and filtered through a no. 5 Whatman paper. The volume of the sample may be varied over wide limits but generally 5 ml. are used. The copper content of the filtrate is determined volumetrically as follows.

10 ml. of the filtrate are acidified with 0.5 ml. acetic acid and approximately 1.0 g. of KI is added.¹ The solution is titrated with standardized thiosulphate, 4 drops of starch solution being added towards the end of the titration. Each ml. of 0.01 *N* thiosulphate is equivalent to 0.28 mg. amino-N. The amount of thiosulphate used is of course corrected for the dilutions involved; i.e. if 5 ml. of sample are used and 10 ml. of filtrate titrated, the volume of 0.01 *N* thiosulphate \times 0.28 gives mg. amino-N per ml. of original material. Where a shortage of material makes it desirable the volume may be reduced and the estimation carried out in 10 ml. volumetric flasks. In this case 6 ml. of the copper phosphate should be used and 2 ml. samples of filtrate titrated with *M*/500 thiosulphate.

RESULTS

Amino-acids

Solutions of each amino-acid were prepared from weighed amounts to contain 70–200 mg. of amino-N per 100 ml. Varying volumes from 2 to 10 ml. were used for the estimations. Table I shows the results obtained.

Table I

Amino-N mg./10 ml.		
Amino-acid	Calculated	Found
Glycine	20.01	20.19
Alanine	20.10	20.19
Aspartic acid	20.02	20.25
Valine	A 9.98	10.32
	B 9.61	10.32
Tyrosine	12.00	12.04
Lysine	A 20.02	9.23*
	B 20.0	9.74
Arginine	9.98	7.72†
Tryptophan	2.12	7.06
+		
Glycine	5.04	12.50
Leucine	2.50	
+		12.90
Aspartic acid	10.00	
Methionine	2.55	12.62
+		
Aspartic acid	9.99	13.09
Phenylalanine	3.03	
+		13.03
Aspartic acid	10.00	

A and B samples from different sources.

* The ϵ group of lysine does not titrate by this method.

† The arginine was impure. The Van Slyke-N and copper values agreed.

¹ See Shaffer & Hartmann [1920–1] concerning the titration of copper iodimetrically.

The first seven acids all form soluble copper salts and present no difficulties. Tryptophan, phenylalanine, methionine and leucine were all estimated after mixture with a known excess of either glycine or aspartic acid. Lysine gives a value equal to half its nitrogen content, since the ϵ group is not determined by this method. Histidine was used as the monohydrochloride. Kober & Sugiura [1913] suggested that histidine formed a complex of the type $(\text{histidine})_3 \text{Cu}_2$ and our results indicate this to be true. Four samples of histidine gave the results shown in Table II. The results found for histidine by the copper method multiplied by 0.75 give the α -amino-N value.

Table II. *Showing the results obtained with histidine monohydrochloride*

Histidine sample	Amino-N mg./10 ml. calculated	Theoretical for $(\text{histidine})_3 \text{Cu}_2$	Found ml. thiosulphate $\times 2.8$
A	9.15	12.2	12.04
A	9.3	12.4	12.39
B	9.5	12.65	12.54
C	9.3	12.40	12.46
D	9.1	12.10	12.24

All the values given in Table I are the means of a large series of determinations. The estimations were made on varying volumes ranging from 2.0 to 10.0 ml., and at the lower end of the range the results were less accurate; exclusion of these results would have given a mean value nearer to the calculated value in most cases.

There appears to be a slight tendency to over-titrate with the thiosulphate, and for accurate work the end-point should be approached somewhat slowly.

Acid hydrolysis of proteins

The method has been used to study the rate of hydrolysis of protein by acids. Experiments on gelatin, zein, edestin and egg albumin were done, using

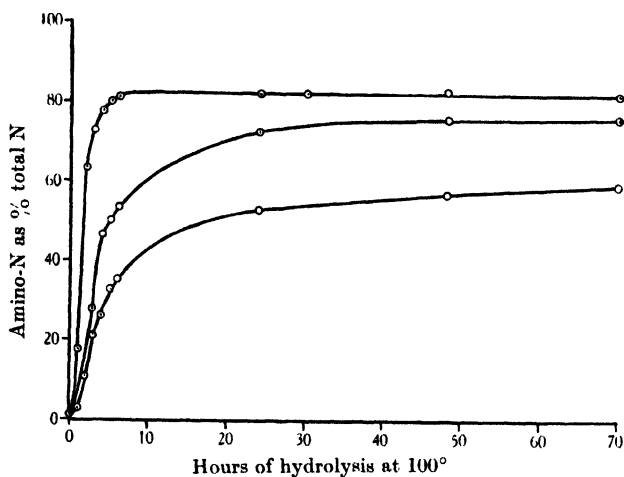


Fig. 1. Showing the hydrolysis of gelatin with 2, 5 and 20% HCl as measured by the copper method.

20% HCl at 100°. The materials were sealed in glass phials to avoid errors due to volume changes during heating. Results showing the course of hydrolysis are given in Figs. 1 and 2 where the amino-N determined by this method is

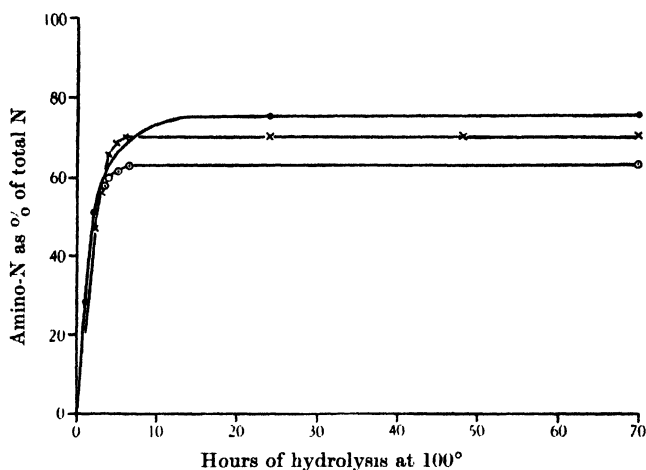


Fig. 2. Showing the hydrolysis of zein, edestin and egg albumin with 20% HCl as measured by the copper method. Zein ●—●. Egg albumin × — ×. Edestin ○—○.

Table III. Showing the amino-acid content of gelatin, total N, and N estimated by the copper and Van Slyke methods

Figures for N represent g /100 g.

Amino-acid	g. present	Total N	Calculated N estimated by	
			Copper method	Van Slyke method
Glycine	25.5	4.75	4.75	4.75
Alanine	8.7	1.36	1.36	1.36
Leucine	7.1	0.76	0.76	0.76
Phenylalanine	1.2	0.10	0.10	0.10
Methionine	1.0	0.09	0.09	0.09
Aspartic acid	3.4	0.36	0.36	0.36
Glutamic acid	5.8	0.55	0.55	0.55
Histidine	0.9	0.24	0.11	0.08
Arginine	9.1	2.90	0.72	0.72
Lysine	5.9	1.18	0.59	0.59*
Proline	19.4	2.36	2.36	—
Hydroxyproline	14.4	1.54	1.54	—
Total	102.4	16.19	13.29	9.36

Theoretical copper value $13.29/16.19 \times 100 = 82\%$. Found 81%.

Theoretical Van Slyke value $9.36/16.19 \times 100 = 58\%$. Found 62.3%.

* Shaken for 4 min.; ϵ group of lysine not included.

Table IV. Showing N distribution figures for gelatin [Van Slyke, 1911-12] and the amounts estimated by the copper and Van Slyke methods

Fraction	% of total N	N estimated by	
		Copper method mg./10 ml.	Van Slyke method mg./10 ml.
Arginine	14.70	3.70	3.70
Histidine	4.48	1.98	1.49
Lysine	6.32	3.16	3.16*
Amino-N of filtrate	56.30	56.30	56.30
Non-amino-N of filtrate			
Proline	14.90	14.90	—
Hydroxyproline			
Total		80.04%	64.65%
		Found 81.0%	Found 62.30%

* Shaken for 4 min.; the ϵ group of lysine not included.

shown as a percentage of the total N. Hydrolysis of gelatin was also carried out with 5 and 2% HCl. Owing to its low solubility the zein was taken into solution in hot acid before it was filled into phials; some hydrolysis had already taken place at this stage. After these proteins had been hydrolysed completely with 20% HCl, the material left in the phials was bulked; estimations were made on the hydrolysate of the total N, and amino-N by the copper and Van Slyke methods. As a matter of interest we have given values deduced from the composition of the protein as given by Jordan-Lloyd & Shore [1938] and from the figures of Van Slyke [1911-12] for N distribution. Those for gelatin are given in full (Tables III and IV) and for the other proteins in summarized form in Table V. The difference between the values obtained by the two methods is large for gelatin owing to the high content of proline and hydroxyproline which are estimated by one method but not by the other.

Table V. *Showing summarized results for edestin and zein hydrolysates obtained by the Van Slyke and copper methods. Amino-N as % of total N*

Material	Method used	Calculated value from data	Found
Edestin hydrolysate	Van Slyke micro-method	57.4*	61.0
		61.58†	
	Copper method	60.0*	62.3
		62.0†	
Zein hydrolysate	Van Slyke micro-method	67.0*	74.6
	Copper method	75.0*	75.7

* Jordan-Lloyd & Shore [1938].

† Van Slyke [1911-12].

Tryptic hydrolysis

For this experiment fibrin was used as substrate and the digestion was carried out with (a) routine trypsin filtrate prepared from ox pancreas according to Cole & Onslow [1916] and (b) similar material treated with acid, based on the method of Northrop & Kunitz [1932], containing largely the proteinase. After 4 hr. digestion at 50° and pH 8, the materials were filtered and determinations made of the total N and amino-N by the Van Slyke and copper methods. Using the Van Slyke method, determinations were made after 4, 8, 16 and 32 min. shaking. Fig. 3 shows the results obtained; for this material at least 20 min. shaking was necessary before a constant result was obtained.

The two methods were also compared on material obtained from casein, deaminized with nitrous acid and afterwards digested with trypsin. The Van Slyke method gave, for 4 min. shaking, 8.95 mg. amino-N per 10 ml. and 10.56 mg. per 10 ml. after 30 min. shaking. The copper value was 10.59 mg. per 10 ml.

For each experiment the copper values quoted are the means of a large series of determinations, all of which agreed closely, in which varying volumes of material were used.

The value of the copper method for studying enzymic hydrolysis is also shown by an experiment on beef muscle. Cooked and well washed muscle was digested for 3 hr. with Merck's trypsin (treated according to Anson & Mirsky [1933]), and then for a further hour with crude untreated trypsin (Merck). Fig. 4 shows the results from copper estimations made during the digestion. After 3 hr. digestion with the purified enzyme (chiefly proteinase) the soluble

total N was 53.3 mg. per 10 ml. of which 9.15 mg. were amino-N (17.3%). 1 hr. after adding the crude trypsin the soluble total N increased to 98.8 mg. per 10 ml. of which 36.3 mg. were amino-N (36.7%).

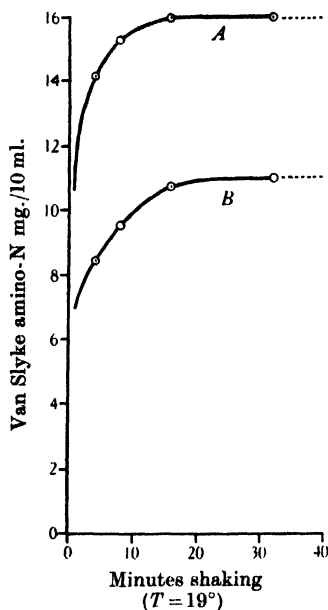


Fig. 3.

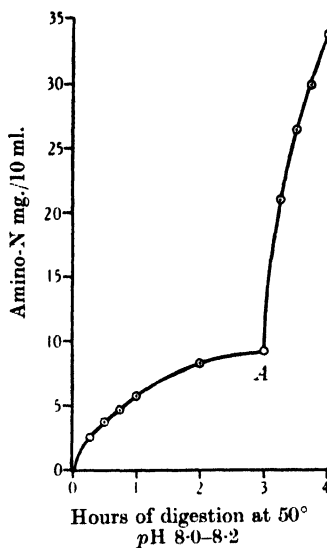


Fig. 4.

Fig. 3. Showing comparative amino-N determinations by the Van Slyke (varying periods of shaking) and copper methods on two tryptic digests of fibrin. *A* = digested with crude trypsin. *B* = digested with proteinase trypsin. The dotted lines show the values obtained by the copper method.

Fig. 4. Showing the amino-N liberated during 3 hr. digestion with purified trypsin. At *A* crude trypsin was added. Determinations by the copper method. Substrate—cooked and washed beef muscle.

SUMMARY

A copper method for determining amino-N, which has been in routine use in these Laboratories since 1935, is described. This method has been found particularly suitable for determining the amino-N liberated by acid or enzyme hydrolysis of proteins. It gives quantitative results for pure amino-acids whose copper salts are soluble, and can be applied to some others which form insoluble copper salts. Some experimental results showing application of the method are recorded.

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CXXXI. PHOSPHATE FRACTIONS IN BARLEY SEEDLINGS

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WORK on the acceleration of plant respiration by the addition of inorganic phosphates has never emerged from the unsatisfactory state which is revealed in Onslow's [1931] extensive summary. The present paper deals with the partition of phosphates in young barley seedlings, and the results of adding or withholding inorganic phosphate supplies. The bearing on seedling respiration is to be dealt with in a subsequent paper.

Methods

Method of cultivation. The paleae and glumes were first removed from the seeds, as it was found that a higher percentage germination could be obtained in this way, and that the seeds germinated more uniformly. The seeds were then soaked for 3 hr. in distilled water at 22.5°. After soaking, the seeds were washed in about a dozen rinsings of autoclaved water and finally in the appropriate culture solution, and placed on the surface of fine sand in a petri dish, the sand being saturated with culture solution. The petri dish and implanted seed were then enclosed in sterilized dark chambers [James & Norval, 1938], and the whole immersed in a thermostat at 22.5°. A continuous current of CO₂-free air was circulated through the chamber. Before entering the chamber, but after being freed from CO₂, the air stream was saturated with water vapour by passing it through a bubbling bottle immersed in the water bath. In this way evaporation from the seedlings and sand in the respiration chamber was cut down to the minimum. The air stream was also passed through a long cotton wool filter to remove spores.

The culture solution was that described by James [1933], and was made up freshly for each experiment from stock solutions which had been sterilized by autoclaving and kept in the dark until required for use; no precipitation occurred, at least until the experiment was actually in progress. In this paper, only "full" phosphate and "no" phosphate solutions are considered.

The sand used to grow the seedlings was purified from all traces of soluble salts by prolonged boiling in HCl followed by aqua regia. The leached sand was then washed repeatedly in distilled water and sterilized by autoclaving. The sterilization was repeated after each experiment, after thorough washing. The seed used in all experiments was "Plumage Archer" obtained from Messrs Sutton and Sons of Reading.

Sampling. While the seedlings were germinating in the respiration chamber, a continuous record of their CO₂ emission was kept. As soon as the chamber was opened up at the end of the germination period, the seedlings were prepared for analysis. They were first washed free from sand and culture solution, and the remainder of the husk and endosperm removed. They were graded at the same time into three grades according to size: grade I restricted to the longest individuals, grade II composed of individuals of length down to about two-thirds of

the maximum length and grade III including the remainder, i.e. all those seedlings under two-thirds of the maximum length. Grade III rarely contained more than 10% of the total number of seedlings, and these were always rejected in sampling for dry weight and analysis. This grading was carried out in order to eliminate, as far as possible, variations between the different samples caused by variation in size of the seedlings. All the samples in any one experiment were selected so that they contained the same proportion of seedlings from grades I and II.

It might be objected that the exclusion of the grade III seedlings from the samples made them incomparable with the whole set of seedlings whose respiration is measured. This procedure has been selected as the lesser of two evils for the following reasons. It has been observed in the present work, that not all the seedlings which are soaked at any one time will germinate simultaneously. A small percentage shows the first visible sign of germination (protrusion of the radicle) about two or three days after the rest. Seedlings formed from these late-germinating seeds are considerably shorter than those from seeds which germinate immediately after soaking, and their physiology may have a corresponding lag; they correspond to the grade III seedlings which are excluded from the samples. They will clearly contribute only a small percentage to the total CO_2 of respiration.

The size of the samples used for extraction varied at different stages. In the preliminary experiments of 1935, the samples contained only 20 seedlings each, two samples being taken from each experiment: the size of these samples was restricted by the size of the chambers in which they were grown. In later experiments, using larger chambers, a larger number of seedlings was grown in each experiment, and two samples each of 60 seedlings were analysed. This was done to reduce the experimental error, which seems largely due to differences between the samples, as will be shown later.

In each experiment, two other samples were taken for dry weight and total P determinations, both determinations being performed successively on each sample. These samples contained 10 seedlings each in the 1935 experiments, and 20 seedlings each in the subsequent experiments; they contained the same proportion of grade I and grade II seedlings as did the samples for extraction. All the results given in the present paper were obtained from the larger samples.

Extraction. Extraction was carried out with trichloroacetic acid, which coagulated all proteins: these could then be centrifuged down [Needham *et al.* 1932; Cori & Cori, 1932]. A somewhat similar technique has been used on plant material by Barrenscheen and co-workers [1927; 1929]. The seedlings were crushed in an ice-cold glass mortar with a little sand. They were then extracted in ice-cold 7.5% trichloroacetic acid for 30 min. in an ice bath, with frequent stirring to facilitate extraction.

A test experiment was performed to ensure that the extraction was complete. It was found that 60 ml. of trichloroacetic acid, followed by a washing in 10 ml. of the same solution, were sufficient for complete extraction, if the extraction lasted for 30 min. Prolonging the period of extraction for a further 30 min. did not result in any increase in the amount of phosphate extracted, but did lead to a slight amount of hydrolysis of esterified phosphate. The increase in inorganic phosphate during the second half hour was about 3%. The possibility of a more rapid breakdown of some of the esterified phosphate will be dealt with in the section on phosphagens which follows, in which it is shown that there is no breakdown of esterified phosphate during the first part of the extraction period. It can therefore be concluded that the slight variations which occurred in the length

of the extraction period in the various experiments to be reported later, are not responsible for the variation in the proportion of inorganic to esterified phosphate.

Fractionation. The trichloroacetic acid extract will contain the following groups of phosphorous compounds, if they are present in the seedlings: inorganic orthophosphate; "labile esters" such as adenylyl pyrophosphate; "resistant esters" such as hexosephosphates, triosephosphates etc; phosphagens such as arginine and creatine phosphates; phytin.

Phospholipins and phosphoproteins will not be extracted in the acid, and they are the main constituents of the "residual P" fraction which is estimated as the difference between the total acid-soluble P and the total P content of the seedlings estimated by incineration.

For simplicity, it will be convenient to state here some conclusions arrived at in the following sections, viz. that there is no phytin nor phosphagen present in the seedlings at this stage of growth. The separation of the remaining three classes soluble in acid is brought about by dividing the extract into aliquots. The total extract is neutralized with saturated NaOH until just alkaline to phenolphthalein. It is then made up to 101 ml. This allows 10 aliquots of 10 ml. each to be removed with a pipette, the extra 1 ml. allowing the last aliquot also to be removed by pipette.

The inorganic orthophosphate can be estimated directly on the extract, by the colorimetric method of Fiske & Subbarow [1925]. It was, however, found better to precipitate the inorganic phosphate by adding some 20% CaCl_2 solution (saturated with CaO), to the neutralized aliquots, and to carry out the estimation of orthophosphate on the redissolved precipitate [Needham *et al.* 1932], because it was not always possible to obtain the crude extract quite clear, even with high centrifuge speeds, and the turbidity gave rise to a distinct yellow tinge in the phosphomolybdate blue, thus rendering comparison with the standard inaccurate. The inorganic orthophosphate determination was done on 4 aliquots. Two other aliquots were made up to *N* with HCl and placed in a boiling water bath for 7 min., being subsequently cooled and neutralized. This mild acid hydrolysis liberates orthophosphate from the "labile esters", and a Fiske & Subbarow determination on these hydrolysed aliquots, therefore, gives the sum of the preformed inorganic orthophosphate, plus the "labile ester" fraction. The "labile ester" fraction is then the difference between this estimate and the inorganic orthophosphate estimate previously obtained.

The remaining four aliquots were incinerated (without calcium precipitation) with H_2SO_4 and H_2O_2 , and the total P content estimated as orthophosphate by the Fiske & Subbarow method. This gives an estimate of the total acid-soluble P. The "resistant ester" content can be determined as the difference between this estimate and the estimate from mild acid hydrolysis, which includes both the other fractions. This will be referred to as "the difference method". The "resistant ester" content can also be determined more directly on the solution left over after precipitation of orthophosphate and pyrophosphate as Ca salts ("solubility method"). The Ca remaining in the solution is first precipitated as oxalate in the presence of acetic acid which keeps other phosphates in solution. The solution is then oxidized with H_2SO_4 and H_2O_2 , and the phosphate content estimated by the Fiske & Subbarow method. It has been found that the same values are obtained whether the "labile esters" have been hydrolysed before the Ca precipitation or not. It follows that the labile esters must be fully precipitated from solution by the Ca precipitation technique. Since the labile esters are precipitated, the method of determining the resistant ester content (by oxidation of the centrifugate after Ca precipitation), is sound, and may be

expected to give reliable results. Table I shows the values for resistant esters obtained by the two methods in two independent experiments.

Table I

	mg. per seedling	
	(1)	(2)
Total acid-soluble phosphate less phosphate after mild hydrolysis	0.0336	0.0214
Phosphate not precipitated by Ca	0.0327	0.0210

RESULTS

In Table II the results of two successive experiments carried out on Dec. 9 and Dec. 16, 1936 are given in full detail. The groups of four estimations represent the four determinations on four aliquots from the same extract. The two groups of four for each experiment represent the two separate extractions carried out on the two comparable samples from the set of seedlings grown in any one experiment.

Table II. *Mg. phosphate per seedling*

	Inorganic	Labile esters	Total acid-soluble	Total phosphate
Dec. 9, 1936				
Extract 1	0.0632	0.0134	0.1004	
	0.0659	0.0119	0.1049	—
	0.0703	—	0.1044	—
	0.0715	—	0.1037	—
Mean	0.0677	0.0123	0.1033	0.1431
Extract 2	0.0629	0.0159	0.1099	—
	0.0627	0.0144	0.1004	—
	0.0616	—	0.1004	—
	0.0618	—	0.1047	—
Mean	0.0622	0.0151	0.1038	0.1441
Dec. 16, 1936				
Extract 1	0.0812	0.0029	0.1140	—
	0.0808	0.0029	0.1147	—
	0.0812	—	0.1141	—
	0.0800	—	—	—
Mean	0.0808	0.0029	0.1143	0.1517
Extract 2	0.0818	0.0081	0.1114	—
	0.0812	0.0059	0.1110	—
	0.0808	—	0.1110	—
	0.0800	—	0.1113	—
Mean	0.0809	0.0070	0.1112	—

In both experiments the "no-phosphate" solution was used, so that there is no difference in treatment between the two experiments. It is obvious by inspection of these figures that the errors incurred in the estimation of inorganic "resistant ester" and total phosphate are small compared with the large differences which occur between seedlings from different, but comparable, experiments. Only in the estimation of the labile esters do the errors of estimation become anything like commensurate with the differences between experiments. It has, all along, been found that the estimation of labile esters involves considerable experimental error; this is largely because they are determined by difference between fractions which are about ten times larger than the labile ester fraction itself.

Another point which is shown by the above table, and is confirmed by the details of other similar experimental results, is that the difference between the mean values obtained from the two separate extractions in any one experiment

is much smaller than the difference between the mean values for different experiments; thus showing that the variation between experiments is not due merely to sampling errors. It is therefore considered that the analytical method adopted is in general adequate, although the method of estimating labile esters needs improvement.

Phytin

The occurrence of phytin in green plants has not been investigated very thoroughly, apart from the Gramineae, in which group its presence seems to be fairly general. Moutuori [1934] has recently demonstrated its presence in the seeds of several tropical cereals, and Goldfiem [1934] found it in a number of tropical African food plants, many of them not belonging to the order Gramineae. Wiaddrowska [1936] demonstrated the presence of phytin in carrots, beans and cabbage plants, and found that treatment with $MgSO_4$ increased the uptake of phosphorus in these plants, and increased the amount of phytin present in the carrot roots. Marimpietri [1933] found that, in various cereals, after the first period of germination (which roughly corresponds to the period under consideration in the present research), the proportion of phytin-P/total P increased. Kolobkova [1936], in investigating the phytase of wheat flour, found that, in the ungerminated seed, 55 % of the total P is present in the form of phytin, but that, on germination, the phytin content falls considerably and other P compounds, especially phosphoproteins and lipins, accumulate.

Phytin is only present in very small quantities in the seedlings used in the experiments to be considered here.

Tests were carried out on a solution of phytin containing 2 mg. per 100 ml. of water. This is about the concentration in which phytin would be present in the trichloroacetic acid extract if all the "resistant ester" phosphate proved to be phytin phosphate. Aliquots of this solution, neutralized till just alkaline to phenolphthalein, were then treated with the $CaCl_2$ solution used for precipitating phosphates. It was found that the solution left over contained absolutely no phosphate, either free or combined, while the precipitate contained the amount of phosphate which would be expected if all the phytin had been precipitated by the $CaCl_2$. Phytin is therefore completely precipitated under the conditions of the routine estimations. If, therefore, phytin is present in the seedlings, it will all be precipitated along with the inorganic phosphate and labile esters. It will not be estimated with either of these fractions, however, since phytin is stable to the comparatively mild hydrolysis which is used to break down the labile esters. Similarly, it will not be estimated as resistant esters after oxidation of the solution left over after Ca precipitation, since it is itself precipitated; but it will be estimated in the total acid-soluble determinations in which the original acid extract is oxidized.

Thus, if phytin is present, we should expect to find a discrepancy between the resistant ester figures obtained by the "difference method" and the "solubility method". Instead of resistant esters = total acid-soluble - (inorg. PO_4 + labile esters) the relation in the presence of phytin would be resistant esters = total acid-soluble - (inorg. PO_4 + labile esters + phytin). Table I gives results in which both estimates of resistant esters are available, and no such discrepancy occurs; so it can safely be concluded that phytin is absent from the seedlings used in the present experiments.

Confirmation was sought by carrying out some phytin estimations on the extract from seedlings, using the iron titration method of Hubner & Stadler [1914]. The difficulty in obtaining a satisfactory end-point, which has been experienced by other workers, renders this method of little value for the precise

estimation of small quantities of phytin. Nevertheless, the results obtained indicate that only minute quantities of phytin could be present in the extracts, viz. not more than 0.0069 mg. per seedling.

Examination of the remains of the endosperm and pericarp removed from seedlings after 5 days' germination, showed that little phytin was present in them after germination of the embryo; 39 of these endosperms and pericarps were ground up and extracted in 20 ml. of 0.3 *N* HCl for 1 hr.; the extract was then filtered, and diluted to 0.16 *N* with water. Titration with FeCl₃ indicated the presence of 0.227 mg. of phytin phosphate (=0.0058 mg./seedling).

The paleae and glumes removed from the seeds before they were germinated were also shown to contain very little phytin. The figures in Table III were each obtained on separate extracts; the husks from about 80 seeds were used for each extract. As a check on the results, two sets of husks were incinerated for total P; the agreement between these figures, and those obtained by titration, indicates that all the P present is estimated by the titration method, and is therefore probably present in the form of phytin.

The total P content of the seeds is about 0.45 mg. of phosphate-P per seed. Thus the husk contains less than 2% of the total P present in the seed.

Table III. *Content in mg. of phosphate per grain*

	Phytin-P (by titration)	Total P (by incineration)
Paleae and glumes (removed before germination)	0.0168	0.0070
	0.0069	0.0068
	0.0049	—
	0.0053	—
	0.0085	0.0069
Endosperm and pericarp (after 5 days' germination)	0.0058	—
Seedlings without endosperm (after 7 days' germination)	0.0069	—

Phosphagens. Two phosphagens are of common occurrence in animal tissues, creatine phosphate and arginine phosphate, and the wide range of distribution of these two compounds has been shown by the work of Needham *et al.* [1932]. An extensive search of the literature has revealed no mention of the occurrence of phosphagens in green plants. It was, however, necessary to determine whether phosphagens were present or not in the seedlings used in the present work, in order that the correct significance might be attached to the various phosphate fractions estimated. Experiments were therefore performed to test whether there were any labile phosphate-forming compounds of the phosphagen type present in the trichloroacetic extract.

By making only one extraction in trichloroacetic acid, and omitting the second washing of the residue, an estimate of the inorganic phosphate present after only 15 min. from the first crushing can be obtained. This does not give time for phosphagens such as creatine and arginine phosphates to break down to any considerable extent, especially as the extraction was performed in an ice-bath. If the solution, with the blue colour developed, is left for about 30 min. after the first reading, the major portion of the phosphagen should have broken down; especially since the Fiske & Subbarow reagents act as catalysts of the hydrolysis. But in the experiments performed along these lines, no increase in the blue intensity, over and above that found in the standard phosphate solution also, was found to occur.

In another type of experiment the extract was divided into aliquots and the inorganic phosphate was precipitated with CaCl_2 at the end of 15 and 60 min. from crushing. No increase in phosphate could be detected after the longer standing. Hence, it seems certain that the seedlings contain no labile phosphagens of the kind found in animal tissues.

Labile and resistant esters. Recent work has demonstrated the highly important role played in yeast fermentation and muscle glycolysis by the compounds of adenosine with phosphoric acid. The P of this group of compounds is estimated by conversion into inorganic orthophosphate by mild acid hydrolysis with N HCl at 100° for 7 min. The P estimated in this way has usually been designated pyrophosphate, since it was originally thought to contain only inorganic pyrophosphate and adenylyl pyrophosphate.

It has now been found that this fraction may also contain adenylic acid, in which the phosphate has no pyrophosphate bond, and so more recent publications refer to the whole group as "easily hydrolysable esters". By contrast, the hexose- and triose-phosphoric esters, together with phosphoglyceric and phosphopyruvic acids, become the "difficultly hydrolysable esters".

In the present paper these two fractions are called "labile esters" and "resistant esters" respectively.

The difficulties attending the estimation of the labile esters have already been stated; because of this difficulty they have not been estimated in every experiment, but only in a selection of experiments representative of the various treatments and ages for which results are available. Table IV shows that a low but definite fraction of the acid-soluble P is present in the labile ester form.

Table IV. *Mg. phosphate per seedling*

	Inorganic phosphate	Labile esters	Total esters	Resistant esters	
				A	B
5-day seedlings on no phosphate solution	0.088	0.0086	0.0364	0.0278	—
	[0.0677	0.0122	0.0356	—	0.0234
	[0.0622	0.0155	0.0416	—	0.0216
	[0.0808	0.0029	0.0335	—	0.0306
	[0.0809	0.0070	0.0303	—	0.0233
	[0.0703	0.0041	0.0372	—	0.0331
	[0.0673	0.0073	0.0337	—	0.0264
	Mean	0.0739	0.0082	0.0355	0.0272
5-day seedlings on full phosphate solution	[0.1064	0.0070	0.0374	—	0.0304
	[0.1056	0.0069	0.0371	—	0.0302
	0.1188	0.0032	0.0359	0.0336	0.0327
	0.1109	0.0048	0.0268	0.0214	0.0210
	Mean	0.1104	0.0055	0.0333	0.0286
7-day seedlings on full phosphate solution	[0.1450	0.0104	0.0150	—	0.0046
	[0.1546	0.0128	—	—	—
	[0.1312	0.0160	0.0318	—	0.0158
	[0.1359	—	0.0259	—	—
	0.1361	0.0100	0.0263	—	0.0163
	Mean	0.1406	0.0123	0.0234	0.0122

Bracketed series were obtained from independent samples from a single crop.

Under resistant esters, column B gives results obtained by the "difference method", and column A those obtained by the "solubility method".

Analysis of variance shows significant variation in all the phosphate fractions. There are significant differences ($P < 0.05$) between each of the inorganic phosphate means. The difference between full and no phosphate is not significant among resistant esters. All other comparisons of means give significant differences.

Stable esters are also present, and usually contain a much larger part of the phosphate, the exception being in the 7-day-old seedlings.

Inorganic phosphate always accounts for the bulk of the acid-soluble phosphate, even in those seedlings which received no external supply. Phosphate feeding results in considerable increases of the inorganic phosphate within the seedling, but this is not followed by a corresponding increase in the esters of either class. The natural inference is that, in these early seedling stages, the phosphate supplied by the reserve tissues is itself sufficient to maintain all the available "phosphate acceptor" in the esterified form. Between the fifth and seventh days the labile esters, unlike the resistant esters, increase. This may be supposed to have been made possible by further synthesis or absorption from the endosperm of phosphate acceptor.

Comparison of root and shoot contents

In a further set of analyses, the seedlings were divided into "root" and "shoot" portions and the phosphate of these contents determined separately. The seedlings were cut across just above the scutellum into two portions, one containing the coleoptile, first leaves and shoot rudiment, and the other containing the roots and scutellum. In each experiment about 80 seedlings, grown on full phosphate solution, were used; and a further 40 seedlings were similarly divided and used for determinations of fresh and dry weight and of total P.

The results of these experiments are given in Table V. The relative bulks of the root and shoot portions, as indicated by fresh weight, are not very different, the root portion being somewhat less than the shoot.

Table V. (a) *mg. phosphate per seedling*; (b) *mg. phosphate in 1 g. fresh wt.*

	Root		Shoot	
	Exp. 27	Exp. 28	Exp. 27	Exp. 28
Fresh wt. (g. per seedling)	0.0810	0.0783	0.1042	0.1053
Inorganic PO ₄	(a) 0.0548 (b) 0.6760	0.0500 0.6383	0.0641 0.6150	0.0609 0.5783
Labile esters	(a) 0.0017 (b) 0.0207	0.0038 0.0485	0.0015 0.0144	0.0010 0.0095
Resistant esters	(a) 0.0116 (b) 0.1427	0.0067 0.0855	0.0216 0.2072	0.0150 0.1424
Total esters	(a) 0.0123 (b) 0.1512	0.0110 0.1402	0.0237 0.2274	0.0158 0.1500
Residual PO ₄	(a) 0.0022 (b) 0.0284	0.0042 0.0536	0.0258 0.2475	0.0258 0.2450
Total PO ₄	(a) 0.0693 (b) 0.8556	0.0652 0.8321	0.1136 1.0899	0.1025 0.9733

All the fractions determined were present in both portions of the plant, the shoot containing rather more phosphate than the root, in the aggregate.

The distribution of phosphate is expressed in Table VI as fractions of the total phosphate.

Table VI

	Root	Shoot
Inorganic phosphate	0.77	0.58
Labile esters	0.04	0.02
Resistant esters	0.14	0.17
Residual phosphate	0.05	0.23
Total phosphate	1.00	1.00

The proportions allotted to labile and resistant esters do not differ greatly between "root" and "shoot"; but a much larger proportion of the inorganic phosphate is transferred to residual phosphate in the "shoot" than in the "root". This is probably related to the bulkier meristem, since the residual phosphate consists mainly of phosphatides and nucleoproteins.

SUMMARY

1. A method is described for the estimation of the various classes of phosphorus compounds present in plant material, and results are given for seedlings of Plumage Archer barley grown for 5 and 7 days in the absence of light on solutions with and without phosphate.

2. Phytin was only present in very small quantities in the husks (paleae and glumes), endosperms or seedlings proper.

3. Phosphagens of the type of creatine and arginine phosphate could not be detected in the seedlings.

4. The inorganic phosphate content of the seedlings was increased by growing the seedlings on culture solution containing phosphate, but this did not result in an increase of either labile or resistant esters.

5. Inorganic phosphate, labile esters and resistant esters all vary with the stage of development.

6. The contents of the root and shoot portions of the seedlings have been determined and compared. There seems to be little difference between the two portions in regard to labile esters and resistant esters. The shoot has a much larger proportion of lipid and protein phosphorus than the root. This is assumed to be associated with the greater proportion of meristematic tissue present in the shoot portion.

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CXXXII. SYNTHETIC GALACTOSE-1-PHOSPHORIC ACID

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In previous papers [Kosterlitz, 1937; 1938] evidence was given which indicated the presence of galactose-1-phosphate in the livers of rabbits assimilating galactose. Attempts to isolate this ester were unsuccessful because its properties were not known in sufficient detail and the yields obtained were small. Therefore it was found necessary to prepare the synthetic ester before continuing the investigations on the naturally occurring ester.

It was impossible to obtain α - and β -tetraacetyl galactose-1-phosphoric acids by phosphorylation of the corresponding 2:3:4:6-tetraacetyl galactoses with POCl_3 in dry pyridine. Cori *et al.* [1937] reported a similar failure with glucose. These workers synthesized barium glucose-1-phosphate by the action of silver phosphate on acetobromoglucose in dry benzene, hydrolysis of tri-(tetraacetyl glucose)-1-phosphate in methyl alcohol containing 4% of aqueous 5N HCl for 16 hr. at 25° and neutralization of the acid with $\text{Ba}(\text{OH})_2$.

By using this method it was found possible to synthesize galactose-1-phosphoric acid [Colowick, 1938; Kosterlitz, 1938] which is non-reducing and very readily hydrolysed by acid, as is the glucose ester. The preparations obtained by both authors, however, still contained impurities: only 92% (Kosterlitz) and 94.5% (Colowick) of the total P were easily hydrolysable and the specific rotations were +81.3° and +91° respectively. A possible explanation of the difference in the specific rotations of the two preparations will be offered in the experimental part.

The present paper deals mainly with the final stages of the purification of the ester. The method of preparing the crude Ba salt was essentially the same as that used by Cori *et al.* [1937] for the synthesis of glucose-1-phosphoric acid. Barium galactose-1-phosphate could not be completely purified by the repeated precipitation of its aqueous solution with ethyl alcohol. Repeated recrystallization of the dibrucine salt from 40 to 85% methyl alcohol increased the percentage of labile P from 86 to 96%. Crystallization as the dipotassium salt, either starting with the crude Ba salt or better with the dibrucine salt, yielded the pure product.²

The dipotassium salt is very soluble in water and only sparingly soluble in aqueous ethyl alcohol from which it crystallizes with $2\text{H}_2\text{O}$. This water of crystallization is easily given off *in vacuo* over P_2O_5 at 100° and less readily at room temperature. The anhydrous salt has $[\alpha]_D^{20} +108.2^\circ$ and the free acid +148.5°. That the dipotassium salt is homogeneous is shown by the fact that fractional crystallization effects no separation into fractions of different specific rotations. Barium galactose-1-phosphate prepared from the dipotassium salt, is readily soluble in water and insoluble in 50% ethyl alcohol; the anhydrous salt has $[\alpha]_D^{20} +92.7^\circ$.

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² Dipotassium glucose-1-phosphate was described by Kiessling [1938].

Galactose-1-phosphoric acid is non-reducing. It is completely hydrolysed by 0.09 *N* H₂SO₄ at 100° in 2 min. The *k* of hydrolysis in 0.25 *N* HCl is 0.89×10^{-3} at 25° and 5.9×10^{-3} at 37°; it is therefore more rapidly hydrolysed than glucose-1-phosphoric acid [Cori *et al.* 1937]. The ester is completely resistant to hydrolysis in 0.2 *N* NaOH at 100° for 30 min. The hexose component can be identified as galactose by the fermentation and methylphenylhydrazone tests.

The method of preparation of the ester and its specific rotation strongly suggest that it is α -galactopyranose-1-phosphoric acid. Acetobromogalactose has a pyranose structure, and the molecular rotation of the ester is of the same order of magnitude as are the molecular rotations of the known α -galactosides of acyclic alcohols (Table I). All known and otherwise unsubstituted β -galactosides are laevorotatory. A similar relationship may be shown to exist between α -glucosides and the glucose-1-phosphoric acid of Cori *et al.* [1937].

Table I*

Substance	$[\alpha]_D$	$[M]$	Reference for $[\alpha]_D$
α -Methylgalactopyranoside	+196.6°	+38200	Riiber <i>et al.</i> [1929]
α -Ethyl galactoside	+186.8°	+38900	Pascu & Ticharich [1929]
α -Propyl galactoside	+179.0°	+39800	Bourquelot & Aubry [1916]
α -Allyl galactoside	+171.7°	+37800	Pascu & Ticharich [1929]
α -Ethyleneglycol monogalactoside	+169.9°	+38100	Bourquelot [1917]
α (?)-Galactose-1-phosphoric acid	+148.5°	+38600	Present synthesis

* The two known α -galactosides of cyclic alcohols have molecular rotations which are higher than those of the acyclic alcohols, viz. phenyl- α -galactoside 55300 and *o*-cresyl- α -galactoside 51000.

EXPERIMENTAL

Tri-(tetraacetyl galactose)-1-phosphate

A solution of 15.5 g. acetobromogalactose [Fischer & Fischer, 1910] in 75 ml. dry benzene was refluxed with 7.5 g. freshly prepared silver phosphate, a calcium chloride tube being attached to the condenser. The reaction was complete in 1 hr. The filtrate from the silver salts was boiled with charcoal and left overnight over CaCl₂ at 0° to remove the last traces of colloidal silver salts. The filtered solution was then evaporated under reduced pressure at 30° until a thick syrup was obtained. Owing to excessive frothing the final evaporation had to be done in a desiccator. The product (yield 11.5 g. = 87.3 % of theoretical) consisted of an easily pulverizable glass, which could not be crystallized. (Found: C, 44.88, 44.65; H, 5.48, 5.37; CH₃CO, 45.2, 45.6; P, 2.60, 2.58 %. C₄₂H₅₇O₃₁P requires C, 46.29; H, 5.28; CH₃CO, 47.4; P, 2.85 %.) P was determined by Lohmann & Jendrassik's [1926] modification of the method of Fiske & Subbarow [1925]. The compound had $[\alpha]_D^{17} = +119.9^\circ$ (*c* = 2.25 in methyl alcohol).

It was possible to prepare this substance in greater quantities than those stated above. The yield was satisfactory, viz. 80–90 % when 60–140 g. of acetobromogalactose were used, but the product was less pure. This, however, did not interfere with the further steps of the synthesis.

Barium galactose-1-phosphate (crude product)

107 g. tri-(tetraacetyl galactose)-1-phosphate were dissolved in 2140 ml. methyl alcohol to which 88 ml. 5 *N* aqueous HCl had been added. The mixture was incubated for 8 hr. at 25° and, after cooling, brought to pH 8.4 with Ba(OH)₂ (phenolphthalein). After the precipitate had been allowed to settle overnight at 0°, it was centrifuged and extracted exhaustively with water. The Ba salt

was precipitated by adding to its solution an equal volume of alcohol. This process was repeated three times. Finally, the Ba salt was washed with 80% alcohol and dried *in vacuo* over P_2O_5 at room temperature. Yield 14.1 g. = 34.7% of theoretical. 85.6% of the total P was hydrolysed by 0.09N H_2SO_4 at 100° in 10 min. $[\alpha]_D$ (for anhydrous salt): +81.8°, $[\alpha]_D$ (calculated for labile P): +95.5°.

Dibrucine galactose-1-phosphate

12 g. Ba salt were dissolved in 30 ml. warm H_2O ; after cooling, 58 ml. N H_2SO_4 were added, the $BaSO_4$ centrifuged off and 23 g. brucine in 30 ml. warm methyl alcohol added. The solution (120 ml.) was decolored with charcoal and, after addition of acetone (360 ml.), allowed to crystallize at 0°. Yield 26.2 g. = 86% of theoretical. A second crop of 1.6 g. was obtained from the mother liquor. The dibrucine salt was subjected to repeated recrystallizations from 40 to 83% methyl alcohol. The analytical values of the Ba salts prepared from the different fractions are given in Table II.

Table II

Procedure	Yield %	Labile P in % of total	$[\alpha]_D$ of anhydrous Ba salt	$[\alpha]_D$ calculated from labile P
Crude Ba salt	—	85.6	+81.8	+95.5°
1st recrystallization, from 83% methyl alcohol:				
(a) 1st crop	68	91	+86.9°	+95.5°
(b) 2nd crop from mother liquor after concentration and addition of acetone	10	59.6	+59.8°	+100°
2nd recrystallization, from 83% methyl alcohol (slow)	85	92.2	+87.5°	+94.9°
3rd recrystallization, from 50% methyl alcohol (rapid)	87.5	96	+89.5°	+93.2°
4th recrystallization, from 40% methyl alcohol (rapid)	93.5	96.5	+90.4°	+93.6°
Pure Ba salt	—	100	+92.7°	+92.7°

From the values given in this table it may be concluded that the repeated recrystallizations of the dibrucine salts purified the crude product to a considerable degree. The last traces of the impurity, however, were not removed by this method. The specific rotation of the impurity was very small in this particular instance.

The low $[\alpha]_D$ of the Ba salt described earlier [Kosterlitz, 1938], viz. +81.3° of the anhydrous salt and +88° when calculated from labile P, is most probably explained by the presence of a laevorotatory impurity. In that instance, tri-(tetraacetyl galactose)-1-phosphate was hydrolysed by a method different from that employed in the present synthesis: it was refluxed for 2½ hr. with dry methyl alcohol without addition of acid. The crude Ba salt (yield 41.5%) was partially purified by two crystallizations as the dibrucine salt. In Colowick's preparation [1938] the impurity appears to have been dextrorotatory. Acid hydrolysis liberated 94.5% of the theoretical quantity of galactose; the $[\alpha]_D$ of the anhydrous Ba salt was +91°, and +96.4° if calculated from labile galactose.

Dipotassium galactose-1-phosphate

To a solution of 11 g. dibrucine salt with 96.5% of the theoretical labile P in 55 ml. water, 10% KOH was added to pH 8.4 (about 12 ml.); the brucine was filtered off and washed. Ethyl alcohol was added to the combined filtrate and washings until the solution became just turbid. From the mixture, which was kept at 0° overnight, a considerable quantity of the dipotassium salt

crystallized out. The solution was again rendered just turbid with alcohol, this process being repeated at 2-hourly intervals until no further crystallization occurred. Yield 3.25 g. = 83 % of theoretical. For analysis, the substance was recrystallized twice from a 5 % aqueous solution by addition of alcohol. Dipotassium galactose-1-phosphate crystallizes with $2\text{H}_2\text{O}$, which are readily given up on drying *in vacuo* over P_2O_5 at 100° and less readily at room temperature. The anhydrous salt is hygroscopic. The dipotassium salt may also be prepared from the crude Ba salt by removal of the Ba with K_2SO_4 , e.g. 1.5 g. crude Ba salt with 85.6 % labile P gave 0.58 dipotassium salt. Yield 50 % of theoretical. (Found for anhydrous salt: C, 22.06, 21.84; H, 3.45, 3.56; P (total), 9.16, 9.06; P (labile), 9.16, 9.12; P (inorganic: Lohmann [1928]), 0; galactose after hydrolysis, 53.4, 53.9 %. $\text{C}_6\text{H}_{11}\text{O}_6\text{PK}_2$ requires C, 21.41; H, 3.30; P (total=labile), 9.22; P (inorganic), 0; galactose, 53.5 %. Found for hydrated salt: P (total), 8.31, 8.32; P (labile), 8.30, 8.35; P (inorganic), 0; galactose, 48.9 %. $\text{C}_6\text{H}_{11}\text{O}_6\text{PK}_2 \cdot 2\text{H}_2\text{O}$ requires P (total=labile), 8.33; P (inorganic), 0; galactose, 48.4 %.) The anhydrous salt had $[\alpha]_D^{18} + 108.0^\circ$ and $[\alpha]_{540}^{18} + 127.5^\circ$ ($c=2.557$ in water); the hydrated salt had $[\alpha]_D^{17} + 98.0^\circ$ and $[\alpha]_{540}^{17} + 116.0^\circ$ ($c=2.375$ in water). The free acid (solution of the dipotassium salt in 0.2 N HCl) had $[\alpha]_D^{18} + 148.5^\circ$ ($c=1.678$).

Fractional crystallization of the dipotassium salt. To a solution of 2.6 g. of this salt in 52 ml. water an equal quantity of ethyl alcohol was added and the mixture kept at 0° for 36 hr. Yield 1.24 g. A second crop of 0.96 g. was obtained after further additions of alcohol. $[\alpha]_D^{18}$ of original salt + 97.7° ($c=1.684$); of 1st crop + 97.5° ($c=2.668$); of 2nd crop + 98.1° ($c=2.754$).

Barium galactose-1-phosphate prepared from the dipotassium salt

0.8 g. barium acetate in 5 ml. H_2O was added to 0.8 g. dipotassium salt in 5 ml. H_2O . The barium galactose-1-phosphate was then precipitated by the addition of 10 ml. alcohol and a few drops of a saturated solution of $\text{Ba}(\text{OH})_2$ in order to bring the pH to 8.4. The Ba salt was precipitated five times, washed with 80 % alcohol and dried *in vacuo*. Since this salt retained varying quantities of H_2O after drying *in vacuo* over CaCl_2 or P_2O_5 at room temperature, the following method was used for the determination of its specific rotation. The P and galactose liberated by acid hydrolysis were estimated and the

Table III

Method of drying and approximate H_2O content	Total P %	Labile P %	$[\alpha]_D$ of anhydrous salt calculated from P content	Galactose after hydrolysis %	$[\alpha]_D$ of anhydrous salt calculated from galactose content	Galactose P	Ba [King, 1932] %
(1) Dried incompletely over P_2O_5 ; $2\frac{1}{2}\text{H}_2\text{O}$	6.99	6.95	+93.1° ($c=2.22$)	41.5	+91.0°	5.95	—
(2) Redried over P_2O_5 ; $\frac{1}{2}\text{H}_2\text{O}$	7.55	7.55	+94.6° ($c=2.53$)	44.9	+92.4°	5.95	—
(3) No. (2) was redissolved, precipitated again, and dried over CaCl_2 ; $1\frac{1}{2}\text{H}_2\text{O}$	7.40	7.37	+92.4° ($c=2.34$)	42.9	+92.4°	5.82	31.6 31.7

Average rotation = $[\alpha]_D^{18}$ (anhydrous Ba salt): + $92.7 \pm 0.5^\circ$.

Average $\frac{\text{galactose}}{\text{P}}$: 5.91; calculated 5.81.

$\frac{\text{Ba}}{\text{P}}$: found 4.29; calculated 4.43.

measured specific rotations corrected by the factors $\frac{\text{P content of anhydrous salt}}{\text{P content found}}$ and $\frac{\text{galactose content of anhydrous salt}}{\text{galactose content found}}$ (Table III).

Hydrolysis of galactose-1-phosphoric acid

(1) *In 0.09N H₂SO₄ at 100°.* 21.91 mg. dipotassium galactose-1-phosphate were dissolved in 25 ml. H₂O with addition of 1.18 ml. 0.1N H₂SO₄ in order to liberate the free acid. To 20 ml. of this solution 2 ml. N H₂SO₄ were added. Test tubes containing about 5 ml. of the ester-H₂SO₄ mixture were immersed in a boiling water bath for 1, 2 and 3 min., and after cooling, galactose and inorganic P were determined: 1 ml. contained 0.796 mg. dipotassium salt with 0.0663 mg. P and 0.386 mg. galactose (Table IV).

Table IV

Time min.	P formed mg./ml.	Galactose formed mg./ml.
1	0.050	0.295
2	0.066	0.396
3	0.066	0.396

The ester was 76 % hydrolysed in 1 min. and completely hydrolysed in 2 min.

(2) *In 0.25N HCl at 25° and 37°.* Dipotassium galactose-1-phosphate was used for these experiments. The calculated amount of H₂SO₄ was added to liberate the free galactosephosphoric acid. Then equal quantities of ester solution and 0.5N HCl were mixed and incubated in a water bath. The galactose formed was estimated by the method of Hagedorn and Jensen. Since the reducing power of galactose is less than that of glucose, a calibration curve was used which was a straight line when the heating period was extended from 15 to 30 min. Care was taken to neutralize the HCl before the estimation. The inorganic P formed was estimated by the colorimetric method of Fiske & Subbarow [1925]. Since a measurable amount of ester was split by the H₂SO₄ present in the reagent, a correction had to be applied. The time which elapsed between the addition of the molybdic-sulphuric acids and the first reading was

Table V

Hydrolysis at 37.0°. Concentration of ester 2.84 m.-molar. Galactose formed			Hydrolysis at 25.0°. Concentration of ester 2.78 m.-molar. Galactose formed		
Time (min.)	%	$k \times 10^3$		%	$k \times 10^3$
15	17.6	5.6		—	—
30	32.7	5.85		6.2	0.93
45	45.1	5.9		—	—
60	55.4	6.0		11.3	0.81
90	69.6	5.55		16.7	0.91
120	80.4	6.35		—	—
Average		5.9			0.88

Hydrolysis at 25.0°. Concentration of ester 4.08 m.-molar.

Galactose formed			Inorganic P formed		
Time min.	%	$k \times 10^3$		%	$k \times 10^3$
50	10.0	0.915		9.9	0.905
110	20.6	0.905		20.3	0.885
180	31.2	0.89		30.6	0.86
255	41.2	0.91		40.0	0.845
Average		0.905			0.875

noted, and two further readings were taken at intervals of 5 min. From the three values the initial value was extrapolated. The k of hydrolysis was calculated from the equation:

$$k = \frac{1}{t_2 - t_1} \log \frac{(a - x_1)}{(a - x_2)} \text{ (Table V).}$$

(3) *In 0.2N NaOH at 100°.* 4 ml. of a 2.672% solution of dipotassium galactose-1-phosphate and 1 ml. *N* NaOH in a sealed test tube were immersed in a boiling water bath for 30 min. After cooling, 1 ml. *N* H₂SO₄ was added and the rotation determined. $[\alpha]_D^{20} = +3.49^\circ$ ($l=2$) calc. $+3.49^\circ$. 1 ml. of this solution was diluted to 19.80 ml., and reducing power and inorganic P were estimated in the usual way before and after hydrolysis in 0.09 *N* H₂SO₄ at 100° for 5 min. The quantities of galactose (0.0072 mg. in 2 ml.) and inorganic P (0.0006 mg. in 2 ml.) found before acid hydrolysis were negligible and within the ranges of error of the methods employed. After acid hydrolysis, the values found per ml. were 0.449 mg. galactose (calc. 0.435 mg.) and 0.0754 mg. P (calc. 0.075 mg.). Therefore, the ester was completely resistant to hydrolysis in 0.2 *N* NaOH at 100° for 30 min.

Examination of the hexose liberated by acid hydrolysis

(1) *Identification of galactose as methylphenylhydrazine.* 47.7 mg. hydrated dipotassium galactose-1-phosphate were dissolved in 0.5 ml. water in a small centrifuge tube; 1.02 ml. *N* H₂SO₄ were added to liberate the free ester and make the solution 0.5 *N* with regard to H₂SO₄. The ester was then hydrolysed for 10 min. at 100°. After neutralization with 1.02 ml. *N* NaOH, 0.05 ml. glacial acetic acid and 0.03 ml. methylphenylhydrazine were added and the mixture left overnight at 0°. The crystals of the hydrazone were centrifuged off, washed in the centrifuge tube with about 2 ml. ice-cold water and then re-crystallized from about 2 ml. ethyl alcohol. The final product was washed with 2 ml. ice-cold alcohol. Yield 34.6 mg. galactose methylphenylhydrazine (95% of theoretical). M.P. 189 (corr.) with decomposition; mixed M.P. 188. Votoček [1921] gave M.P. 190°.

(2) *Identification of galactose by fermentation analysis.* The method employed was described in an earlier paper [Kosterlitz, 1937]. For the analysis of the unhydrolysed ester a solution containing 0.874 mg. hydrated dipotassium galactose-1-phosphate with 0.423 mg. galactose per 2 ml. was used while for the ester hydrolysed by 0.09 *N* H₂SO₄ at 100° for 10 min. a solution containing 0.728 mg. dipotassium salt with 0.352 mg. galactose per 2 ml. was employed (Table VI).

Table VI

Treatment	Reducing power of 2 ml. unhydrolysed ester in mg. galactose		Reducing power of 2 ml. hydrolysed ester in mg. galactose	
	Calc.	Found	Calc.	Found
Before fermentation	0	0	0.352	0.358
After fermentation with <i>S. Ludwigii</i> (not fermenting galactose)	0.423	0.420	0.352	0.351
After fermentation with galactose- adapted <i>S. cerevisiae</i> Froberg	0	0	0	0

These experiments prove that the hexose component of the ester is galactose; the increase in reducing power of the unhydrolysed ester after treatment with *S. Ludwigii* is due to the liberation of galactose by phosphatase.

SUMMARY

The synthesis and properties of α (?)-galactose-1-phosphoric acid are described.

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CXXXIII. KETOSIS IN THE HYPOPHYSECTOMIZED RAT

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It is generally agreed that the injection of anterior pituitary extracts will increase ketosis in animals. Burn & Ling [1930] originally demonstrated that these extracts increased ketonuria in rats fed on fat and later Anselmino & Hoffmann [1931] showed that there was an increase in blood acetone after injection of similar extracts. These results have been amply confirmed by others and have led to the suggestion that there is a factor present in the anterior pituitary which plays some part in fat metabolism. It has not yet been decided, however, whether the effects that are produced are due to a specific factor for fat metabolism, as suggested by Magistris [1932] and by Anselmino & Hoffmann [1934], or whether they may be the result of one or more of the other known factors in the anterior pituitary, as has been suggested by Shipley & Long [1938].

Since anterior pituitary extracts increase ketosis, it would be expected that hypophysectomy would cause a reduction in ketonuria and a decrease in the blood acetone bodies. Rietti [1933] has shown that the ketonuria in dogs which have been rendered diabetic either by pancreatectomy or by the administration of phlorhizin is less in hypophysectomized animals than in those with the pituitary intact.

The purpose of the present work was to investigate the effects of hypophysectomy on the ketosis produced in rats by a diet of fat.

Methods

The animals used were young females of the London strain of Wistar rat. Females were chosen because they are known to be more susceptible than male animals to ketosis. Rats weighing 120–160 g. were hypophysectomized under ether anaesthesia by the technique of Smith [1927], slightly modified. At 10–20 days after operation they were placed in metabolism cages, fed on a diet consisting of butter with the addition of a 3% salt mixture, as described by Wigglesworth [1924], and their urine was collected in 24-hr. samples under toluene. The glass collecting vessels were washed with distilled water and the urine and washings made up to a volume of 20–25 ml. At the completion of the experiment, the animals were bled by heart puncture under chloroform anaesthesia. Control animals received similar treatment. The total acetone bodies were determined in blood and urine by Van Slyke's gravimetric method with Denigès' reagent [Peters & Van Slyke, 1932], modified for the smaller quantities of material, i.e. 10 ml. urine and 2 ml. blood.

The hypophysectomized animals were very sensitive to cold and in some of the earlier experiments, when the metabolism cages were in a laboratory the temperature of which was not controlled, the animals became cold and moribund

after a few days on the fat diet. Therefore, both hypophysectomized and control animals were kept in the animal room at a temperature of 75° F., which was controlled by a thermostat.

Results

Ketonuria only was investigated in our first group of experiments. In normal rats on the fat diet there was a variable but definite excretion of acetone bodies, but in the hypophysectomized animals on the same diet there was never more than a trace of ketonuria. It had been shown previously [Anderson & Anderson, 1927] that the injection of adrenaline caused an increase in the ketonuria of young rats fed on a fat diet. Therefore, adrenaline was administered subcutaneously to the control and experimental animals in doses of 0.15 mg. per rat on the 4th or 5th day of fat feeding. Our normal animals showed a definite increase in ketonuria after the injection of adrenaline, but the hypophysectomized animals showed no increase whatever.

In four hypophysectomized rats and their controls the blood acetone bodies were estimated at the end of the experiment. Three of these are shown in Figs. 1-3.

In Fig. 1, the daily excretion of total acetone bodies in the urine is plotted for a hypophysectomized rat and its control, and the levels of blood acetone bodies at the end of the experiment are shown by the vertical columns. It will be seen that while the control rat showed a considerable ketonuria, starting on the 3rd day and rising to 65 mg. on the 4th, the hypophysectomized rat did not excrete more than 3 mg. even after 5 days on the fat diet. By contrast, the level of acetone bodies in the blood at the end of the 5th day was nearly the same in both animals, namely, 55 mg./100 ml. in the hypophysectomized animal, as compared with 54 mg. in the control. Another hypophysectomized rat, which was maintained on a fat diet for 7 days, did not excrete any appreciable amount of acetone bodies in the urine, and on the 7th day blood acetone bodies were 25 mg./100 ml.

The results after adrenaline are shown in Figs. 2 and 3. In Fig. 2 the control animal shows a large excretion of acetone bodies, rising to 78 mg. after the injection of adrenaline, while the hypophysectomized animal excreted only traces, i.e. less than 1 mg. per day. The level of acetone bodies in the blood at the end of the 5th day was high in both rats, but higher in the hypophysectomized rat than in the control—88 mg./100 ml. as compared with 63 mg. In the other experiment shown (Fig. 3), in which adrenaline was injected on the 6th day, the control animal showed a slight ketonuria rising to 19 mg. on the 3rd day, falling to 3 and 4 mg. on the 4th and 5th days respectively and rising to 20 mg. after adrenaline, while the hypophysectomized rat excreted only traces. Here again both animals showed a ketonaemia, though of less degree, and the level in the hypophysectomized rat (45 mg.) was greater than that in its control (23 mg.).

It is of interest to note that after the injection of adrenaline glycosuria occurred in the controls, but not in the hypophysectomized rats.

The hypophysectomized rats all had hypoglycaemia at the end of the experiment, with blood sugar levels of 30-40 mg./100 ml., while the control animals showed a less severe reduction in blood sugar, which varied between 42 and 67 mg./100 ml.

DISCUSSION

Our results show that the hypophysectomized rats had no appreciable ketonuria after being fed on a diet of pure fat for as long as a week, whereas the control animals excreted variable but definite amounts of acetone bodies. These

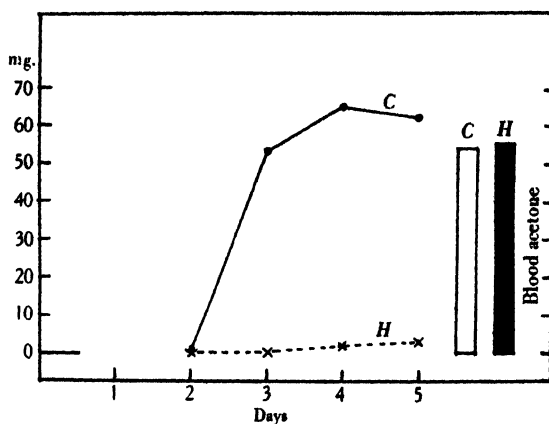


Fig. 1. Abscissa=days. Ordinate=mg. acetone bodies (acetone + acetoacetic + β -hydroxybutyric). Broken line *H*=urinary excretion by hypophysectomized rat. Continuous line *C*=urinary excretion by control. Black vertical column *H*=blood acetone bodies mg./100 ml. in the hypophysectomized rat. White column *C*=blood acetone bodies in the control.

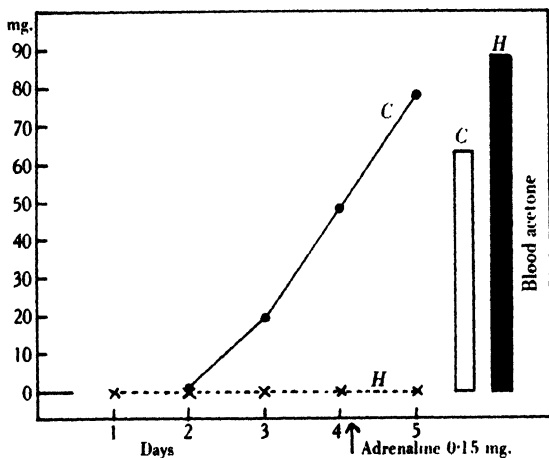


Fig. 2. Abscissa, ordinate etc. as in Fig. 1.

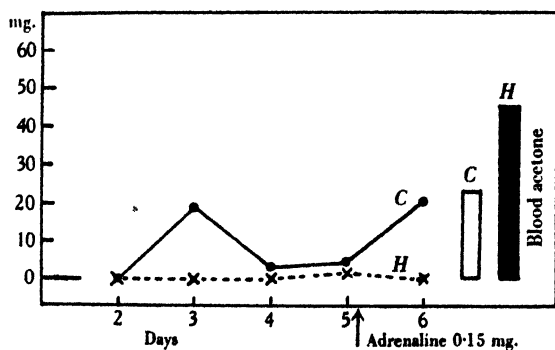


Fig. 3. Abscissa, ordinate etc. as in Fig. 1.

results agree with those of Rietti [1933], referred to above. The subcutaneous injection of adrenaline produced a considerable increase in the ketonuria of the normal rats, as in the previous experiments reported by Anderson & Anderson [1927]. In our hypophysectomized rats, however, the injection of adrenaline had no apparent effect on the excretion of acetone bodies. It should be noted that Russell & Cori [1937] reported that the effect of subcutaneous injection of adrenaline on carbohydrate metabolism was less in the hypophysectomized than in the normal rat, whereas intravenous injection produced the same effect in both.

The absence of ketonuria in the hypophysectomized animals might be explained by the removal of a "ketogenic factor" in the anterior pituitary. The blood acetone bodies, however, instead of being decreased, were actually higher than in the corresponding control animals, whether adrenaline had been administered or not. These results are not necessarily contradictory to the hypothesis of a pituitary "ketogenic factor", because the high level of blood acetone might not have been reached had even small amounts of acetone been excreted in the urine.

The apparent inability to excrete acetone bodies after hypophysectomy must be due to some alteration in renal function, either to a general failure, or to a specific change in the threshold for these bodies. In the dog, hypophysectomy appears to produce a temporary decrease in the concentrating power of the kidney. White & Heinbecker [1938] reported that in hypophysectomized dogs there was a rise in blood urea from 20 to 40 mg./100 ml. at 20 days after the operation, followed by a fall towards normal, and that the urea and creatinine clearances were decreased by nearly half at first, then rose towards normal after a few weeks. Their operations were performed by the ventral approach through the palate, thus avoiding damage to the hypothalamus. In this respect their operations were similar to our own in which damage to the hypothalamic region was excluded.

We have no evidence as to whether a fall in the concentrating power of the kidney for urea etc. occurs in rats after hypophysectomy. Urinary output was not measured in our experiments, but there was no obvious difference in the urinary output of the hypophysectomized and control animals. In a previous publication we reported that the blood inorganic phosphate of the rat was considerably decreased after removal of the pituitary [Anderson & Oastler, 1938]; this decreased concentration of phosphate would not be expected if there were a severe degree of impairment of renal function.

In our opinion, a failure in kidney function of the order found by White & Heinbecker [1938] is not sufficient to account for the almost complete failure of the hypophysectomized rat to excrete acetone bodies.

Shipley & Long [1938] have shown that the urinary excretion of acetone bodies in rats does not rise above 5 mg. in 24 hr. until the blood level has reached a value of about 25 to 30 mg./100 ml. Beyond this point they found that the excretion rose sharply. They consider that this represents a threshold level for β -hydroxybutyric acid and acetoacetic acid; acetone had already been shown not to be a threshold substance [Widmark, 1920; Briggs & Shaffer, 1921]. In our experiments, the normal animals showed the usual marked variation in the degree of ketonuria which seems to depend on the degree of ketonaemia. In two of the normal animals (Figs. 1, 2), there was a great excretion of acetone bodies and in them the blood acetone was raised far above the threshold value. In the animal with a smaller degree of ketonuria (Fig. 3), the blood acetone was at the threshold value. Some of our other normal animals settled down to an excretion

of only a few mg. per day, and in the only one of these in which blood acetone bodies were estimated, only traces were found. In the hypophysectomized animals the threshold value was exceeded in each case, and yet there was no appreciable ketonuria. Russell & Cori [1937] showed that the threshold for sugar was raised in rats after hypophysectomy. They demonstrated this by the administration of adrenaline intravenously and by the infusion of glucose. They concluded that the excretion of sugar in the urine was not a good index of the adrenaline action because of the alteration of kidney function in the hypophysectomized rat, whereby, although the blood sugar was higher than in the controls, the excretion was considerably lessened.

Neufeld & Collip [1938] observed that in fasting adrenalectomized rats treated with potent ketogenic pituitary extracts there was no ketonuria, but that there was a ketonaemia equal to that of normal rats similarly treated, provided that the animals were in good condition; they suggested that the failure of the adrenalectomized rats to show ketonuria was due to a raised kidney threshold for acetone bodies.

The results of our experiments would suggest that the threshold for acetone bodies is also raised after hypophysectomy. Whether this is directly due to lack of the pituitary, or can be accounted for by atrophy of the adrenal cortex from lack of the adrenotrophic factor cannot be decided on the evidence available.

Our results prove that the presence or absence of ketonuria cannot be accepted as an indication of the degree of ketosis in the hypophysectomized rat.

SUMMARY

1. Hypophysectomized female rats excreted only traces of acetone bodies in the urine when fed on a diet of fat for as long as a week, nor did they show any increase in ketonuria after the subcutaneous injection of adrenaline. Control animals showed a variable but definite ketonuria, which was increased by the injection of adrenaline.

2. At the end of the period of fat-feeding, blood acetone bodies were higher in the hypophysectomized than in the control rats and were above the normal threshold level.

3. It is suggested that the absence of ketonuria in the hypophysectomized rats is due to an increase in the renal threshold for acetone bodies after the removal of the pituitary.

We are indebted to the Medical Research Council for a grant for expenses to one of us (E. G. O.).

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CXXXIV. THE ESTIMATION OF COCARBOXYLASE (VITAMIN B₁ DIPHOSPHATE ESTER) IN BLOOD

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It is now generally believed that vitamin B₁ must first be converted into its diphosphate ester before it acts as a coenzyme necessary for the degradation of pyruvate [see Banga *et al.* 1939]. Estimations of cocarboxylase in blood in health and disease are obviously of great interest, particularly since nearly 90 % of the vitamin in the blood is in the phosphorylated form. Simola [1932] showed that the amount of cocarboxylase in the brain tissue of rats was reduced in avitaminosis; Ochoa & Peters [1938] and Westenbrink & Goudsmit [1938] have published figures for other animal tissues. Apart from a preliminary account of the work in this paper, presented before the Physiological Society in March [Goodhart & Sinclair, 1939], no estimations upon human blood have been published. Both Heyns [1939] and Hennessy & Cerecedo [1939] have also described methods applicable to blood.

Ochoa & Peters [1938] have reported a method for the quantitative determination of vitamin B₁ and cocarboxylase in boiled tissue extracts, based upon the observation of Ochoa [1938] that vitamin B₁ markedly stimulates the decarboxylation of pyruvic acid by alkaline-washed yeast in the presence of cocarboxylase. We have applied their method, slightly modified, to the estimation of cocarboxylase in blood, but we have not found it satisfactory for the estimation of blood vitamin B₁.

Method

Dry baker's yeast was freed from cocarboxylase by rapid washing at room temperature with 0.1 *M* Na₂HPO₄ (1 g. yeast with three lots of 50 ml. phosphate) and once with distilled water. It was then washed twice with 50 ml. acetone and dried *in vacuo*. The activity of this preparation remained constant for at least 3 weeks if kept *in vacuo* when not in use. The production of CO₂ from pyruvic acid, in presence of the yeast preparation (as a source of carboxylase) and excess vitamin B₁, was measured in Warburg manometers. To each 25 ml. bottle were added in order: sufficient phosphate buffer (*pH* 6.2) to bring the total volume to 3.0 ml.; 1.0 ml. blood containing 0.2 % neutral potassium oxalate; sufficient HCl to bring the *pH* to 6.2; 0.1 ml. of a solution containing MgCl₂, MnCl₂ and CaCl₂ (0.1 mg. Mg, 3 μ g. Mn, 0.96 mg. Ca); 0.2 ml. vitamin solution (containing 10 μ g. vitamin B₁); 1.0 ml. yeast suspension (100 mg. of the yeast preparation in 0.1 *M* phosphate buffer, *pH* 6.2). The CaCl₂ was omitted from control flasks that did not contain blood. After equilibrating in the bath at 28° in an atmosphere of nitrogen, the reaction was started by tipping in from the side-bulb 0.2 ml. sodium pyruvate (*pH* 6.2, containing 5 mg. pyruvic acid). All determinations were done in duplicate.

Ochoa & Peters [1938] showed that if nearly maximum amounts of vitamin B₁ were added to washed yeast and pyruvic acid, the amount of CO₂ produced

was proportional to the amount of cocarboxylase added. We have found that the height and shape of the curve obtained by adding known amounts of cocarboxylase to the system vary sufficiently with different batches of the yeast preparation to necessitate determining the curve with each new batch (Fig. 1). The evolution of CO_2 was measured 30 min. after tipping in the pyruvate, and the amount of cocarboxylase in the blood was obtained by comparing the difference in evolution of CO_2 between the flasks containing blood and the control flasks with the appropriate standard curve.

For reasons mentioned below, the flasks containing blood were placed for two minutes in a boiling water bath immediately after adding the solution containing MgCl_2 etc. This undoubtedly destroys many substances that would interfere, and it does not destroy the cocarboxylase. The anticoagulant used is important, since both sodium citrate (0.5%) and potassium oxalate (0.2%) inhibit the system. We have used 0.2% potassium oxalate and have added CaCl_2 to precipitate it after the blood has been added to the phosphate buffer. Too much CaCl_2 must not be added, because concentrations of 0.4% decrease the activity of the system. We have checked the method we finally adopted by adding blood rapidly to the phosphate buffer without using any anticoagulant and without adding CaCl_2 . Ascorbic acid (in added amounts up to 0.1%) and nicotinic acid (up to 0.04%) had no adjuvant action; 0.1% nicotinic acid stimulated the system. No effect was observed with urea (up to 0.2%) or with glucose (up to 0.1%). We have tried the effect of addition of various salts; we do not believe that inorganic substances in blood affect our system in presence of the ions mentioned above. Many other substances were tried by Ochoa & Peters [1938]. The presence in blood of vitamin B_1 monophosphate ester or of the pyrimidine or thiazole components of the vitamin would not affect our estimations because we are working in presence of nearly maximum amounts of vitamin B_1 . Adding varying amounts of cocarboxylase to blood produces the expected results [see also Ochoa & Peters, 1938]. A very important factor is the careful control of pH. All the flasks start at pH 6.2 and the flasks containing blood remain at this pH throughout the experiment but the control flasks become very slightly more acid (pH 6.1 to 6.0) after 1 hr. Probably owing to this, rather higher cocarboxylase values are found for blood in the second half hour than in the first, since the activity drops off faster in the control flasks than in the flasks containing blood. For this reason we have used the values obtained at the end of the first half hour.

It will be shown below that blood converts free vitamin B_1 into cocarboxylase. If sterile blood be kept at room temperature for 24 hr. there may be a slight increase in the amount of cocarboxylase; at 0° there is no increase. This is not a likely source of error if the blood is tested as soon as possible after collection or is kept at 0° when necessary, particularly since there is very little free vitamin B_1 in blood.

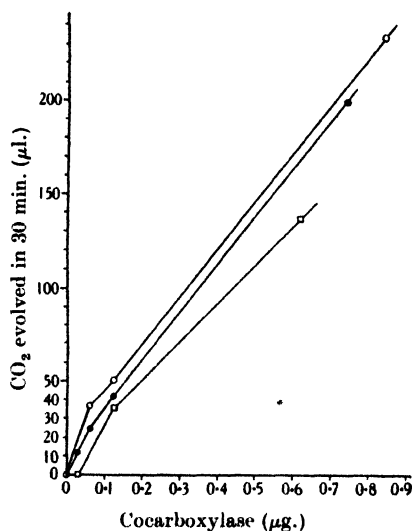


Fig. 1. Effect of cocarboxylase upon the activity of alkaline-washed yeast.

RESULTS

(1) *Effect of heating blood*

One of us [Sinclair, 1938] found that there were only traces of free vitamin B₁ in unheated blood, using the method of Meiklejohn [1937] for the determination (this method determines both unesterified vitamin and cocarboxylase). Sinclair concluded that practically all the vitamin B₁ in blood was combined, probably with protein. With the method described in this paper we have uniformly obtained appreciable evolution of CO₂ when using unheated blood. Experiments indicate, however, that this is not due to cocarboxylase in the blood. When small amounts (0.13 μ g.) of cocarboxylase are added to samples of human blood that have been previously heated to different temperatures, the percentage recovery varies with the temperature to which the blood is heated, as shown in Fig. 2, curve 1. Curves 2 and 3 in the same figure represent the volume of gas produced by two human bloods over the same temperature range, without added cocarboxylase. If the gas produced by unheated blood represented entirely CO₂ evolved by the action of the cocarboxylase in blood upon pyruvic acid, curves 2 and 3 should approximate in shape to curve 1. Actually they differ considerably. This difference is emphasized by curve 4, which represents the average % yield of cocarboxylase that would obtain in the case of the two bloods used for curves 2 and 3 if it were assumed that the gas produced by unheated blood represented exactly a 100 % yield of cocarboxylase. As there is negligible destruction of the vitamin when boiled for two minutes at pH 6.2, we conclude from these curves that most of the gas evolved by unheated blood is not due to the action of cocarboxylase.

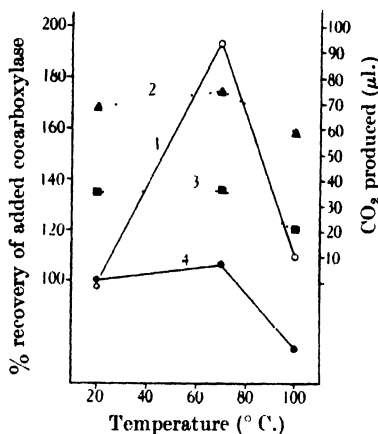


Fig. 2. Effect of heating upon the evolution of CO₂ by blood, and upon the apparent yield of added cocarboxylase.

Table I. *The effect of heat on the cocarboxylase activity of blood*

	μ l. CO ₂ evolved in 30 min.					
	Normal pigeon			Avitaminous pigeon		
	Not heated	70°	100°	Not heated	70°	100°
In bath at once	48.9	77.0	52.3	58.5	37.5	23.2
In bath after 1 hr.	7.0	54.1	55.2	21.3	42.9	31.8

In Table I are given similar results obtained with the bloods of two avitaminous and two normal pigeons. The figures in the lower column were obtained with bloods that had previously been standing for 60 min. in air and at room temperature, in the presence of phosphate buffer (pH 6.2) and Mg, Mn and Ca salts. The volumes of CO₂ evolved by these bloods when not heated were considerably less than the volumes evolved by the corresponding bloods that had not received this extra hour of equilibration. Such differences were not apparent in the case of the corresponding boiled bloods. The boiled avitaminous bloods gave significantly lower values than did the boiled normal bloods, whereas the

results obtained with the unheated bloods did not indicate which were the avitaminous and which the normal birds.

That this activity of unheated blood is no function of vitamin B₁ is also indicated by the fact that it is shown in the absence of yeast, under which conditions the activity is not enhanced by the addition of either cocarboxylase or vitamin B₁. It is depressed to some extent by the addition of alkaline-washed yeast.

From the above facts we cannot entirely exclude the possibility of some cocarboxylase acting in unheated blood but we may safely conclude that this is increased by denaturation of the blood by heating. The increased gas production by bloods that have been heated at 70° for 2 min. compared with boiled bloods is due to the liberation of an adjuvant factor at this temperature, as shown by curve 1, Fig. 2.

In all subsequent experiments we have used oxalated blood that has been boiled for 2 min. in the presence of phosphate buffer (pH 6.2) and Mg, Mn and Ca salts.

(2) *The amount of cocarboxylase in normal human blood*

Estimations of cocarboxylase have been done on 36 different samples of blood taken at least 2 hr. after a meal from 26 presumably normal adult males aged between 20 and 40 years. This group comprised 17 research workers, 4 resident hospital physicians and 5 medical students. These individuals were living on ordinary unrestricted diets and were not receiving any vitamin supplement.

The results are shown in Fig. 3, where each point in column 1 represents a determination upon one of the 36 samples. The average values for the 26 individuals range from 4.5 to 12.0 $\mu\text{g.}$ per 100 ml., the mean is 7.0 and the standard deviation of the individual observations is 2.1. In columns 2, 3 and 4 are given the results of estimations on three different subjects, the samples being collected at intervals of one week to several months. It will be seen that the variation in samples collected from one subject at different times is as great as the variation of the whole group of different subjects. The factors that cause this variation in the amount of cocarboxylase in the blood of an individual are not known and require further study.

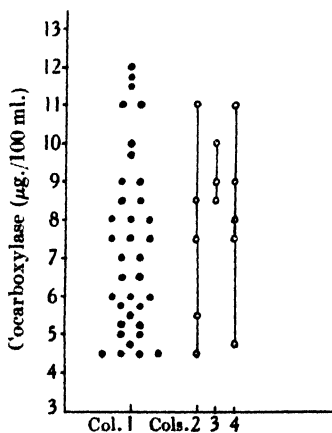


Fig. 3. Amount of cocarboxylase in human blood, and its variation in the blood of three subjects.

(3) *The amount of cocarboxylase in the blood of various animals*

The following values (mean in $\mu\text{g.}/100 \text{ ml.} \pm \sigma$) have been obtained for the blood of different animals, including the results mentioned in the last section.

Human (26)	7.0 \pm 2.1
Ox (5)	5.7 \pm 1.5
Pigeon (5)	20.2 \pm 9.8
Avitaminous pigeon (5)	5.6 \pm 0.8

The cocarboxylase in the blood of the pigeons deficient in vitamin B₁, which were all in opisthotonos, is significantly lower than the normal (the difference

between the two means divided by the estimated standard error of this difference is 3.3), and its level is very consistent; this suggests that symptoms of deficiency may arise at a particular level of cocarboxylase in the blood.

(4) *The distribution of vitamin B₁ and cocarboxylase between the plasma and blood cells*

Meiklejohn [1937] stated that about 20% of the total vitamin B₁ in human blood was present in plasma; but as he used as anticoagulant a concentration of potassium oxalate (0.3%) that may be haemolytic, his value may have been too high. However, one of us (H. M. S.), in unpublished experiments in which care was taken to avoid haemolysis, obtained an average value of 14%. We have studied the distribution of both cocarboxylase and vitamin B₁ between plasma and cells. Table II summarizes results with human, ox and pigeon plasma; cocarboxylase is expressed in $\mu\text{g./100 ml. plasma}$. As we have not found the method of Ochoa & Peters [1938] to be sufficiently sensitive for the quantitative determination of vitamin B₁, we have tabulated the results of these estimations merely as $\mu\text{l. CO}_2$ produced in 30 min. by 1 ml. plasma, in presence of 2 $\mu\text{g. cocarboxylase}$ and no added vitamin B₁. The small positive values for cocarboxylase which were obtained in 9 out of the 17 cases tabulated were all associated with partial haemolysis before or during separation of the plasma. Plasma uncontaminated in this manner gave uniformly negative results. If there is no cocarboxylase in plasma there should be none in normal cerebrospinal fluid. In Table II are tabulated the results on 9 samples of c.s.f. Only one positive result was obtained, and this in a fluid that was markedly xanthochromic.

Table II. *The amount of cocarboxylase and vitamin B₁ in plasma and cerebrospinal fluid*

Human plasma		Human c.s.f.		Ox plasma		Pigeon plasma	
Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$	Cocarb. $\mu\text{g./100 ml.}$	Vitamin B ₁ $\mu\text{l.CO}_2$
0.5	72	0	—	1.5	—	0	—
—	53	0	—	0	—	3.0	18
0	59	0	—	0	—	—	—
0	37	5.0	—	0	—	—	—
—	—	0	44	—	14	—	—
—	—	0	43	2.3	35	—	—
—	—	0	43	1.5	10	—	—
—	—	0	48	3.5	17	—	—
—	—	0	33	2.0	29	—	—
—	—	—	—	0	—	—	—
—	—	—	—	1.0	—	—	—
—	—	—	—	1.0	21	—	—
—	—	—	—	0	16	—	—

In contradistinction to the results obtained with nearly maximum amounts of vitamin B₁, the gas evolved with a constant amount of cocarboxylase (2 $\mu\text{g.}$) has been consistently more with both plasma and spinal fluid than in the controls, indicating the presence of vitamin B₁ in these fluids.

In Table III the cocarboxylase values ($\mu\text{g./100 ml. blood}$) obtained with ox and pigeon whole bloods are compared with the values given by the erythrocyte and leucocyte layers of the same bloods. These cells were separated from the whole blood by centrifuging at 2500 rev./min. for 1½ hr. Fractionation was not complete, the leucocytes in the "leucocyte layers" of the ox bloods numbering

Table III. *Amount of cocarboxylase in different layers of blood ($\mu\text{g.}/100\text{ ml.}$)*

Source	Ox				Pigeon	
	1	2	3	4	1	2
Whole blood	6.5	5.5	5.0	6.1	19.0	31.7
R.B.C. layer	7.3	14.0	5.0	11.5	67.0	50.6
W.B.C. layer	27.5	23.0	17.0	36.5	14.5	9.5

from 123,000 to 246,000 per $\mu\text{l.}$ with erythrocyte counts of 1,000,000 to 5,760,000. The erythrocytes varied from 11,120,000 to 16,000,000 per $\mu\text{l.}$ in the "erythrocyte layers" of the ox bloods, with leucocyte counts of 1200 to 6700 per $\mu\text{l.}$ Similar variations occurred with the pigeon bloods but, owing to technical difficulties, accurate leucocyte counts are not available for these bloods.

Despite this imperfect separation of cells the results shown in Table III clearly indicate that there is a much larger amount of cocarboxylase in the mammalian leucocyte than in the corresponding erythrocyte. The results on pigeon blood are interesting in that here this difference does not occur. This difference and its explanation are more clearly shown in Fig. 4, where curves 1

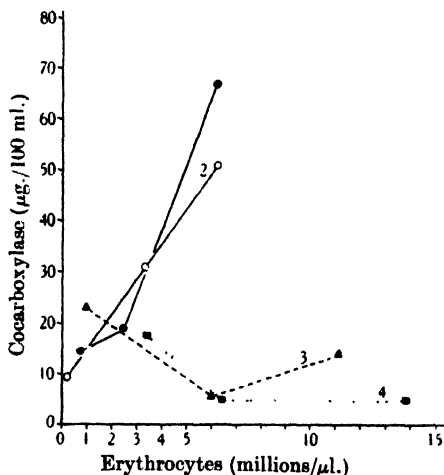


Fig. 4. Effect of the cell count upon the cocarboxylase content of blood.

and 2 represent cocarboxylase values obtained with pigeon bloods and curves 3 and 4 the corresponding values of two ox bloods. The smallest erythrocyte count plotted for each blood was obtained with the "leucocyte layer" and represents, therefore, the highest leucocyte count for that blood. In the same way, the highest erythrocyte represents the smallest leucocyte count. The middle values of each curve were obtained with the whole bloods. It can be seen that the cocarboxylase content of pigeon blood tends to vary directly with the erythrocyte count except, perhaps, with very small erythrocyte counts when the presence of large numbers of leucocytes may tend to flatten the curve. It therefore seems likely that, in pigeon blood, the erythrocyte contains as much cocarboxylase as the leucocyte and the increased cocarboxylase content of whole pigeon blood over ox blood is due entirely to this large erythrocyte content.

In the case of ox blood, the rapid fall in cocarboxylase values with decreasing leucocyte counts, despite much greater increases in the number of erythrocytes, indicates the relative importance of these two types of cells in determining the amount of cocarboxylase in mammalian bloods. The tendency of the cocarboxylase value to increase again with further concentration of erythrocytes, despite a slight reduction in leucocytes over the normal value, shows that the mammalian erythrocyte is not entirely devoid of cocarboxylase. In the blood used for curve 4 (Fig. 4) the cocarboxylase content of the "erythrocyte layer" was the same as that of the whole blood. As there were 7,420,000 more erythrocytes and 4700 less leucocytes in the former, it follows that one leucocyte contained as much cocarboxylase, in this instance, as 1600 erythrocytes. This figure is merely illustrative: it is not intended to be taken as more than a very rough approximation of the relative amounts of cocarboxylase in these two types of cells, as neither the method of determining cocarboxylase nor the technique used in doing the cell counts permits exact calculations of this nature.

The amounts of gas evolved by the same samples of blood in the presence of 2 μ g. cocarboxylase are listed in Table IV. In every instance, except one, the figure is largest with the "erythrocyte layers", while the "leucocyte layers"

Table IV. *Amount of vitamin B₁ in different layers of blood*

	μ l. CO ₂ evolved in 30 min.				
	Ox				Pigeon
	1	2	3	4	
Whole blood	53	16	26	—	37
R.B.C. layer	77	29	14	61	76
W.B.C. layer	54	15	27	47	36
Plasma	35	8	17	29	18

give values no greater than those given by the corresponding whole bloods. As the total number of cells in the "erythrocyte layers" is much greater than, and the total number in the "leucocyte layers" is only slightly less than, those in the whole bloods, the results are entirely compatible with the assumption that the blood vitamin B₁ identified in this way readily diffuses and is adsorbed. It should be noted that the values given by pigeon blood, in this instance, are of the same order of magnitude as those given by the ox bloods. The vitamin B₁ thus determined represents only a fraction of the total blood vitamin B₁. We have been able to increase the yield of apparent vitamin B₁ from sheep and ox serum by incubating the serum at pH 3.0 for 7 hr. at 38° in the presence of a concentration of 0.25% of recrystallized pepsin (Table V). This increased yield has been obtained without any change in the amount of cocarboxylase.

Table V. *Effect of pepsin on the amount of vitamin B₁*

	μ l. CO ₂ evolved		
	Not treated	Boiled pepsin	Pepsin
Ox serum	21	34	73
Sheep serum	4	10	35

(5) *Formation of cocarboxylase from vitamin B₁ by blood cells*

The finding that the nucleated blood cells contained large amounts of cocarboxylase, while the non-nucleated mammalian erythrocyte contained very little, and the plasma none at all, suggested to us the possibility that the nucleated blood cells possessed the ability to form cocarboxylase from vitamin B₁. To test this point we incubated samples of 1 ml. oxalated blood for 1 hr. in air at 38° in the presence of 10 µg. vitamin B₁, 0.1 mg. Mg, 3 µg. Mn, 0.96 mg. Ca and 0.5 ml. phosphate buffer pH 6.2. Under these conditions we were not able to demonstrate any synthesis in normal pigeon, ox, or human bloods—a result that is in accord with that of Ochoa, namely, that tissues saturated with cocarboxylase will produce negligible synthesis even under optimum conditions (pH about 8.5 and in an atmosphere of O₂).

The incubation under the same conditions of avitaminous pigeon blood with 10 µg. vitamin B₁ resulted in the synthesis of a considerable amount of cocarboxylase (Table VI). The increase in cocarboxylase that resulted from incubation

Table VI. *Effect of incubation with vitamin B₁ on cocarboxylase content of blood (µg./100 ml.)*

Source	Avitaminous pigeon			Human myeloid leukaemia	Human lymphatic leukaemia
	1	2	3		
Control	5.0	6.0	5.5	124.0	17.5
Incubated with 10 µg. vit. B ₁	25.0	29.0	19.5	174.0	14.5

brought the total amount up to within the limits found for the blood of normal pigeons (see results for whole pigeon blood in Table III).

In Table VI are also listed the results on two human bloods, one from a case of myeloid and the other from a case of lymphatic leukaemia. The erythrocyte count in each instance was approximately the same, 1,500,000 per µl. The total leucocyte count in the myeloid leukaemia was 730,000 per µl. and in the lymphatic leukaemia 360,000 per µl. The much larger amount of cocarboxylase in the former case cannot be accounted for by the difference in cell count alone. It would appear that cells of the myelogenous series contain a great deal more cocarboxylase than do those of the lymphatic series. Also, incubation, in the case of the myeloid leukaemic blood, resulted in a still further increase in cocarboxylase, whereas incubation of the lymphatic leukaemic blood produced no synthesis. One cannot conclude from this that the synthesis of cocarboxylase is a function restricted to those blood cells that originated in the bone marrow, since the state of saturation (before incubation) of the lymphocytes with cocarboxylase in this case of lymphatic leukaemia is not known. More work on this problem is necessary. At present it is safe only to say that those nucleated blood cells that originate within the bone marrow are the most active elements of the blood in synthesizing cocarboxylase from vitamin B₁.

From the above facts, it seems quite probable that the cocarboxylase contained within the mammalian non-nucleated erythrocyte represents the residuum of that acquired by this cell in its nucleated form, while still within the bone marrow.

DISCUSSION

The fact that the cocarboxylase of the blood is contained entirely within the blood cells makes it seem unlikely that the vitamin is carried to the tissues by the blood in the phosphorylated form. It is much more probable that the readily

diffusible vitamin B₁ (or vitamin B₁ monophosphate), which has been demonstrated to be present throughout the blood, is the direct source of both the vitamin B₁ and the cocarboxylase of the fixed tissue cells. The liver and (to a lesser extent) muscle and brain [Ochoa & Peters, 1938] and the kidney [Westenbrink & Goudsmit, 1938] can phosphorylate vitamin B₁. We have shown that the nucleated blood cells that originate in the bone marrow are active in this respect. It seems probable that the phosphorylation is a function possessed by all nucleated animal cells. The liver and kidney appear to be most active in this respect; the former probably phosphorylates vitamin B₁ partly for storage, unesterifying the cocarboxylase when necessary to replenish the blood vitamin B₁. In the kidney phosphorylation may be essential for the reabsorption of the vitamin in the tubules; no cocarboxylase normally appears in the urine.

The inclusion of all the cocarboxylase within the blood cells depreciates to a varying extent the value of total blood vitamin B₁ or of cocarboxylase determinations as a means for determining the presence or absence of deficiency of vitamin B₁ in any individual with a blood dyscrasia. Thus an individual with myeloid leukaemia, with a leucocyte count of 200,000 or more, may be found to have a total blood vitamin B₁ content about six times the normal value, although the plasma vitamin B₁ level may be much less than normal, and the individual may actually suffer from a deficiency of this vitamin. Conversely, an individual with marked anaemia and leucopenia may have a subnormal value for total vitamin B₁ in the blood, with a normal plasma level and no symptoms referable to a deficiency of vitamin B₁.

Admittedly, variations in the cell count, whether red or white, must be large before appreciably affecting the blood level of cocarboxylase. An increased blood value can readily be observed with an increase in the polymorphonuclear leucocyte count of 20,000 or with an increase in the erythrocyte count of 2,000,000 per μ l.

SUMMARY

1. A reliable method for the rapid determination of cocarboxylase in small samples (2 ml.) of oxalated blood has been described.

2. Reasons are given for believing that all the cocarboxylase present in blood is in a combined form.

3. All the blood cocarboxylase is found within the blood cells, the polymorphonuclear leucocytes containing more than the lymphocytes and the non-nucleated erythrocytes containing least.

4. The nucleated blood cells can phosphorylate vitamin B₁; reasons for believing that this is a function common to all nucleated animal cells are stated. Probably the mammalian non-nucleated erythrocytes obtain their cocarboxylase while in the nucleated form within the bone marrow.

5. Evidence is presented for a freely diffusible form of vitamin B₁ in the blood. In addition, there probably exists a form in combination with protein that is not cocarboxylase and is present in serum.

6. The vitamin is probably carried to the tissues as free vitamin B₁ or its monophosphate ester, and not as cocarboxylase.

7. The results of 36 estimations of cocarboxylase in samples of blood from 26 normal male adults are presented. The mean value is 7.0 μ g./100 ml., $\sigma=2.1$.

8. The limitations of estimations of total vitamin B₁ in blood as indices of the presence or absence of deficiency of the vitamin are discussed.

We are very grateful to Prof. R. A. Peters for his interest and advice throughout this work.

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CXXXV. PYRUVATE OXIDATION IN BRAIN

VI. THE ACTIVE FORM OF VITAMIN B₁ AND THE ROLE OF C₄ DICARBOXYLIC ACIDS

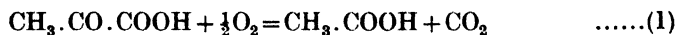
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THE object of this paper is to present a final proof that the form of vitamin B₁ active in pyruvic acid oxidation is the pyrophosphate and to show that C₄ dicarboxylic acids are an essential part of this system. In so doing not only is the hypothesis of the German workers [Lohmann & Schuster, 1937] proved, but also the importance of recent Hungarian work [Szent-Györgyi *et al.* 1936] fully substantiated. We may also consider that we know now the main facts about the biochemistry of vitamin B₁.

The discovery by Lohmann & Schuster [1937] that cocarboxylase is the pyrophosphate of vitamin B₁, strongly suggested that the phosphoester was the form in which the vitamin is active in the oxidation of pyruvate in animal tissues. It was pointed out in a previous paper [Ochoa & Peters, 1938, 1] that there were two main lines of evidence to support this view: (1) The presence of cocarboxylase in the tissues and the ability of tissues to phosphorylate vitamin B₁; (2) the alleged activity of cocarboxylase in "catatorulin" tests with slices of avitaminous pigeon's brain. Further indirect support was given by the experiments of Lipmann [1937] who showed that the oxidative decarboxylation of pyruvic acid according to reaction (1) or its dismutation [Krebs & Johnson, 1937] according to reaction (2), by alkaline-washed preparations of lactic acid bacteria (*Bacterium Delbrückii*), was catalysed by the pyrophosphate but not by free vitamin B₁. More recently Barron & Lyman [1939] have confirmed this for various strains of gonococcus and staphylococcus.



As regards the presence of cocarboxylase in animal tissues, Ochoa & Peters [1938, 1] showed that in rat's and pigeon's tissues there is cocarboxylase but little free vitamin B₁; brain contains not more than 10% at most of the total vitamin as free vitamin (or monophosphate) and the same is the case with liver.¹ Similar results were obtained by Westenbrink & Goudsmit [1938] using a different method. The amount of cocarboxylase was much reduced in the B₁-avitaminous conditions. Injections of vitamin B₁ led to marked synthesis of cocarboxylase in the liver of avitaminous animals [Ochoa & Peters, 1938, 1; confirmed by Westenbrink & Goudsmit [1938], who also showed this in the

¹ Recent observations show that the vitamin in pigeon's breast muscle not present as cocarboxylase is very likely monophosphoric ester. The whole of the thiochrome-yielding substances in boiled extracts is precipitated by Pb and no thiochrome can be extracted by isobutyl alcohol [Ochoa, unpublished results].

kidney]. The vitamin is also readily phosphorylated to cocarboxylase by the liver *in vitro*; with brain and muscle the synthesis was much less [Ochoa & Peters, 1938, 2].

Direct proof of the activity of vitamin B₁ pyrophosphate in animal tissues was however not available, since Peters [1937] failed to confirm Lohmann & Schuster's [1937] contention that cocarboxylase produced equal "catatorulin" effects to vitamin B₁ using avitaminous pigeon's brain brei. That the pyrophosphate is really the active form we have now proved by the use of two different types of finely ground brain preparations I and II. Whereas the brei previously used showed a much larger effect with free vitamin B₁, preparation I is activated by cocarboxylase but not by free vitamin B₁, and preparation II, while being well activated by cocarboxylase, only responds to relatively large amounts of vitamin B₁. With preparation II the activity of vitamin B₁ is due to its synthesis to cocarboxylase, as is also the case with the brei. With both preparations I and II the oxidation of pyruvate is found to be catalysed by the system of C₄ dicarboxylic acids.

Experimental methods

Brain slices or brain brei were used as in previous experiments upon the catatorulin test [Peters, 1938]. Finely ground brain preparations (dispersions) were prepared as follows.

(I) The fresh brain was thoroughly ground up in an ice-cold mortar and successively extracted by grinding with small volumes of ice-cold (1) Ringer-phosphate pH 7.3, (2) phosphate buffer (M/10) pH 7.3, and (3) and (4) distilled water. After each extract the whole was centrifuged; the extracts were combined and finally mixed with the solid residue.

(II) The brain, ground as in I, was extracted with ice-cold Ringer-phosphate and the mixture pressed through muslin.

In either case a fine suspension is obtained which can be accurately pipetted into the manometer bottles, the dispersion being the final addition. One pigeon brain (cerebrum + optic lobes) can make as much as 21 ml. of which 2.0 can be taken per bottle.

Cocarboxylase was determined by the method of Ochoa & Peters [1938, 1] in boiled samples. In some cases, when the amounts of tissue used were small or the dilution high, the contents of the bottles were transferred quantitatively into centrifuge tubes and spun sharply; the residue was then mixed with the appropriate amount of water and boiled in the usual way. All the cocarboxylase is present in the solid residue.

Reduction of oxaloacetic to malic acid was determined by the method of Massart [1939].

Both Barcroft and Warburg manometers have been used, with air or O₂ as gas. Temp. 38°.

Preparations

Vitamin B₁ chloride hydrochloride. Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

Cocarboxylase. One pure specimen from Prof. K. Lohmann (Berlin). This was occasionally used in the experiments but mainly employed to standardize the other preparation, a synthetic specimen prepared by the method of Weijland & Tauber [1938] by Messrs Merck and Co., U.S.A. The purity of this specimen as tested in this laboratory proved to be 65%. All figures given in tables, etc. are calculated for pure cocarboxylase.

Vitamin B₁ monophosphate. A synthetic specimen prepared in this laboratory by Mr L. A. Stocken (vitamin B₁-free; 2% cocarboxylase).

Crystalline Na pyruvate as prepared in this laboratory was used throughout. The fumarate was Kahlbaum's Na salt and the Na oxaloacetate a pure specimen from the Biochemical Laboratory of Szeged, Hungary.

Presentation of results.

The ideal method of presenting results in tables probably does not exist. In the catatorulin tests with slices and brei, the previous use of rates expressed as $\mu\text{l. O}_2/\text{g.} \times \text{hr.}$ (wet wt.) has been followed here; in the experiments with dispersions it has been thought more informative to express the actual O₂ uptake in $\mu\text{l.}$ for the sample taken, as it is easier so to relate it to the amount of cocarboxylase placed in the bottle.

1. EXPERIMENTS WITH BRAIN SLICES AND BREI

Doubt was thrown upon the view of Lohmann & Schuster [1937] that vitamin B₁ pyrophosphate was the active form of the vitamin in tissues owing to the failure to confirm the equal activity in the catatorulin tests. As the experiments by the German workers were done with brain slices, we here record in detail in Table I (A) three out of eight experiments of our own using small amounts of vitamin B₁ and approximately equivalent amounts of cocarboxylase. In no case is there a catatorulin effect of cocarboxylase approaching that of the free vitamin.

Table I. *Comparison of catatorulin effects of vitamin B₁ and cocarboxylase using slices of pigeon's cerebrum*

Figures represent rate of O₂ uptake in $\mu\text{l. g. tissue} \times \text{hr.}$ for each period (0.018 M pyruvate).

Successive periods (min.) ...	15	15	30	30	Average cataractin effect	
					First 30 min.	Last 60 min.
(A) Small equivalent amounts of cocarboxylase (0.5 $\mu\text{g.}$) and vitamin B ₁ (0.35 $\mu\text{g.}$)						
1. No addition	2680	2630	2202	1798	—	—
Cocarboxylase	2592	2500	2245	1802	- 109	+ 23
Vitamin B ₁	2808	2910	2810	2550	+ 204	+ 680
2. No addition	2322	2010	1742	1037	—	—
Cocarboxylase	2156	2060	1888	1265	- 80	+ 188
Vitamin B ₁	2403	2620	2578	2177	+ 345	+ 988
3. No addition	1500	1400	1076	956	—	—
Cocarboxylase	1440	1355	1252	1155	- 52	+ 188
Vitamin B ₁	1823	1690	1752	1773	+ 306	+ 746
In all cases cocarboxylase or vitamin B ₁ are triplicates; no addition, duplicate.						
(B) Large equivalent amounts of cocarboxylase (7.5 $\mu\text{g.}$) and vitamin B ₁ (5 $\mu\text{g.}$); the latter is much in excess of maximum vitamin B ₁ needed.						
4. No addition	2740	2650	2420	1970	—	—
Cocarboxylase	3730	3800	3633	3140	+ 1070	+ 1192
Vitamin B ₁	3670	3740	3567	3170	+ 1010	+ 1173

Figures are the mean of duplicates, in all cases carefully shuffled.

That the apparently positive results of Lohmann & Schuster can be imitated by using excessive amounts of cocarboxylase is shown by Exp. 4 of Table I (B). Here a large catatorulin effect is produced which is identical with that of vitamin B₁.

Two further brei experiments were done at very alkaline reaction ($pH\ 8.2$). Table II shows that even here cocarboxylase is not so active as vitamin B_1 .

Table II. *Comparison of catatorulin effects of vitamin B_1 and cocarboxylase with pigeon's brain brei at alkaline reaction ($pH\ 8.2$)*

Cocarboxylase $5\ \mu g.$; vitamin $B_1\ 2\ \mu g.$ Figures represent average rate of respiration ($\mu l./g.$ tissue \times hr.) for the period 30–90 min. ($0.018\ M$ pyruvate).

	O_2 uptake	Catatorulin increase
No addition	759	—
Cocarboxylase	885	+ 126
Vitamin B_1	1226	+ 467
Cocarboxylase + vitamin	1237	+ 478

Hence it is definite that with brei, under all conditions, or with slices, cocarboxylase is less active in the catatorulin test than free vitamin B_1 . When this fact was first clear with brei, it was suggested as one explanation [Peters, 1937] that a permeability factor might be responsible. Since then Dr H. M. Carleton, in as yet unpublished work, has shown that nerve cells are largely destroyed in preparing the brei, though the nuclei are mainly intact. This made the permeability explanation less acceptable; nevertheless the work now to be considered shows that it is the right one. Using the dispersions of brain tissue, evidence has been obtained that the failure of brei to react with the combined form of vitamin B_1 is due to failure to reach the active centre.

The effects of C_4 dicarboxylic acids on the O_2 uptake of brain brei (pigeon) in the presence of pyruvate are generally small. Table III shows the result of such an experiment with fumarate and also the effect of malonate [cf. Greville, 1936; Weil-Malherbe, 1937] which produces here only slight inhibition. Whereas the net O_2 uptake of fumarate alone is $42\ \mu l.$ the net O_2 uptake of pyruvate is increased in presence of fumarate by $310\ \mu l.$ These effects are much more marked in the brain dispersions.

Table III. *Effects of fumarate and malonate on the oxidation of pyruvate in brei from normal pigeon's brain*

Concentration of pyruvate $0.025\ M$.

	$\mu l.$ O_2 uptake per g. tissue in 1 hr. with		
	No addition	Fumarate [$0.005\ M$]	Malonate [$0.024\ M$]
Tissue (about 200 mg.)	960	1002	726
Tissue + pyruvate	1760	2110	1360
Net O_2 uptake of pyruvate	800	1110	634

Increase of O_2 uptake of pyruvate in presence of fumarate $310\ \mu l.$ (+ 39 %). Inhibition by malonate of net O_2 uptake of pyruvate $166\ \mu l.$ (– 21 %).

2. EXPERIMENTS WITH BRAIN DISPERSIONS

A. *Pyruvate oxidation and the role of C_4 dicarboxylic acids*

The O_2 uptake of the brain dispersions, in the presence of pyruvate, is markedly stimulated by addition of fumarate. That this effect is catalytic is shown by the fact that the extra O_2 uptake in the presence of both pyruvate and fumarate is much higher than the sum of the extra uptakes with pyruvate and fumarate separately. The experiment of Table IV shows this very clearly. With

Table IV. *Effect of fumarate on the oxidation of pyruvate in pigeon's brain dispersion (type II)*

Each sample contained 2 ml. enzyme (about 220 mg. brain). Concentration of pyruvate 0.025 *M*.

	μ l. O ₂ uptake in 1 hr. with	
	No addition	Fumarate [0.005 <i>M</i>]
Enzyme alone	154	177
Enzyme + pyruvate	274	384
Net O ₂ uptake of pyruvate	120	207

Increase of O₂ uptake of pyruvate in presence of fumarate 87 μ l. (+ 73 %).

these preparations a high respiration is obtained with pyruvate + fumarate which however falls off rather rapidly after 30–40 min. at 38°, probably owing to instability of some of the enzymic components of the pyruvate oxidation system.

The inhibition by malonate of the pyruvate oxidation is much more marked in the brain dispersions than in the brei and reaches values about 70 % (Table V); this is as high as found by Szent-Györgyi *et al.* [1935] for the inhibition of respiration in muscle dispersions. Curiously enough the residual respiration of the brain dispersion is not so strongly inhibited (*cf.* Table V).

Table V. *Malonate inhibition of pyruvate oxidation in pigeon's brain dispersion (type II)*

Each sample contained 2 ml. enzyme (about 220 mg. brain). Concentration of pyruvate 0.025 *M*.

Time min.	μ l. O ₂ uptake				Net O ₂ uptake of pyruvate (3-1)	Net O ₂ uptake of pyruvate with malonate (4-2)	% inhibition by malonate of pyruvate oxidation
	Enzyme (1)	Enzyme + malonate [0.024 <i>M</i>] (2)	Enzyme + pyruvate (3)	Enzyme + malonate + pyruvate (4)			
10	48	28	104	48	56	20	64
30	85	60	230	93	145	33	78
50	114	91	310	148	196	57	71

The better results obtained with the brain dispersions, as compared with brei, are probably due to the higher degree of division of the tissue in the former which would allow the C₄ acids (which are rather firmly bound by the enzymes [*cf.* Banga, 1937]) to diffuse more readily into the surrounding fluid, their concentration thus easily falling below the optimum required for their catalytic activity; also the malonate should reach the succinodehydrogenase more easily.

The negative results of Schoen & Gerard [1937], who used "solutions" of rabbit's brain in water (brainsols), are difficult to explain. With lactate as substrate they found neither a catalytic effect of succinate nor an inhibition by malonate. Although their lactate oxidation might stop short at pyruvate, possibly owing to low pyruvate dehydrogenase and to the low salt content, yet one would expect the lactic dehydrogenase, being a coenzyme I system, to be activated by the C₄ dicarboxylic acids. Recent experiments with dialysed brain dispersions [Banga *et al.* 1939] show still more clearly the indispensability of these acids for the pyruvate oxidation in brain. Further, the reduction of added oxaloacetate to malate which rapidly takes place in muscle preparations [Szent-Györgyi, 1937; Massart, 1939] is also observed in brain brei as well as in our brain dispersions as is shown in Table VI. The experiments of Table VI were done by

incubating 500 mg. pigeon's brain brei suspended in 1.5 ml. Ringer-phosphate (pH 7.3) or 1.5 ml. dispersion II (500 mg. brain) with 0.013 *M* oxaloacetate for 5 min. at 38°. The total volume was 4 ml. The samples were deproteinized with 0.5 ml. 10% trichloroacetic acid, oxidized with 0.1 *N* KMnO_4 , and the neutralized solution was treated with fumarase, the fumarate formed from malate being titrated with 0.01 *N* KMnO_4 [Massart, 1939].

Table VI. *Reduction of oxaloacetate in brain*

No.	Enzyme	Fumaric acid found after fumarase	Calculated malic acid (as fumaric) after fumarase
		mg.	mg.
1	Brei	0.218	0.87
2	Dispersion II	0.216	0.86
3	Dispersion II	0.200	0.80
Average		0.21	0.84

The total fumarate was therefore 1.05 mg. as fumaric acid or 1.22 mg. as malic acid. Massart [1939] found in muscle under identical conditions 1.80 mg. malic acid formed from oxaloacetate, thus brain forms about 70% of the amount that muscle forms.

B. Activity of cocarboxylase

Cocarboxylase in very small amounts strongly stimulates the oxidation of pyruvate in the salt dispersions of avitaminous pigeon brain. Free vitamin B_1 is either quite inactive or very little active in dispersions of type I but shows some activity in those of type II. Typical experiments with dispersion I are given in Table VII and Fig. 1; in Table VII one out of four similar experiments with a dispersion (type I) prepared from brain of "rice-fed" pigeons (showing no symptoms) is also quoted (Exp. 7). Experiments with type II dispersions are shown in Tables VIII, IX and XI and Figs. 2 and 3. It may be seen that with this type of dispersion free vitamin B_1 shows small but definite effects.

Table VII. *Effects of vitamin B_1 and cocarboxylase on the O_2 uptake of dispersions (type I) from avitaminous pigeon brain in the presence of pyruvate (0.018 *M*) and fumarate (0.004 *M*)*

1 ml. enzyme (140–190 mg. brain) to 2.5 ml. with additions. Air; 38°.

No.	Condition of birds	$\mu\text{l. O}_2$ uptake in 30 min. with					Increase $\mu\text{l. O}_2$ 30 min. with cocarboxylase
		No addition	Vitamin B_1 ($\mu\text{g.}$)	Cocarboxylase ($\mu\text{g.}$)	Vitamin B_1 + cocarboxylase		
1	Avitaminous	149	(2) 135	(3.2) 194	186		+ 45
2	"	120	(4) 122	(1.0) 198	209		+ 78
3	"	100	—	(0.16) 118	—		—
		—	—	(0.53) 133	—		—
		—	—	(1.6) 133	—		+ 33
4	"	107	(20) 119	(1.6) 157	—		+ 50
5	"	68	(4) 71	(1.6) 117	117		+ 49
		—	(20) 70	—	—		—
6	"	87	—	(0.15) 150	—		—
		—	—	(0.2) 156	—		—
		—	—	(0.3) 153	—		—
		—	—	(0.5) 163	—		+ 76
7	Rice-fed (no symptoms)	143	(10) 144	(1.6) 150	153		+ 7

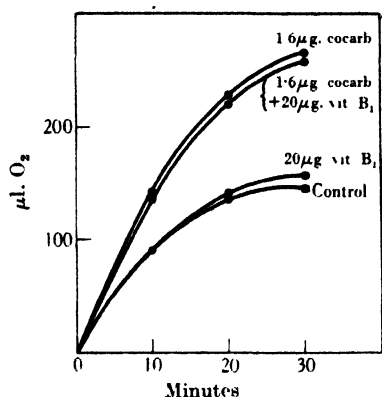


Fig. 1. Comparison of the effects of vitamin B₁ and cocarboxylase on the oxidation of pyruvate (0.018 *M*) in dispersions (type I) from avitaminous pigeon brain, in the presence of fumarate (0.004 *M*). (Samples as in Table VII.)

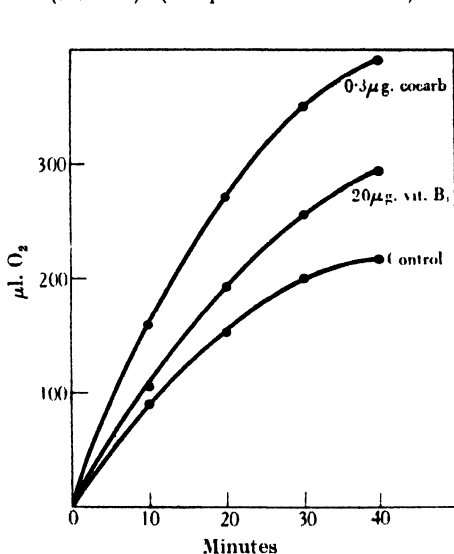


Fig. 2.

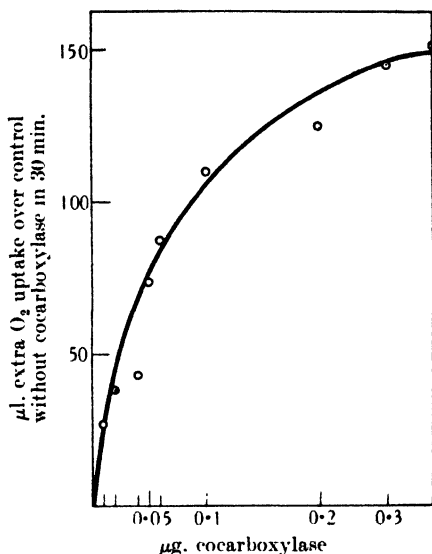


Fig. 3.

Fig. 2. Comparison of the effects of vitamin B₁ and cocarboxylase on the oxidation of pyruvate (0.027 *M*) in dispersions (type II) from avitaminous pigeon brain, in the presence of fumarate (0.005 *M*). (Samples as in Table VIII.)

Fig. 3. Relation between extra O₂ uptake and cocarboxylase concentration in dispersions (type II) from avitaminous pigeon brain. 1.5 ml. enzyme to 2 ml. with additions. 0.027 *M* pyruvate, 0.005 *M* fumarate. O₂, 38°. 320 mg. brain.

Table VIII. *Effects of vitamin B₁ and cocarboxylase on the O₂ uptake of dispersions (type II) from avitaminous pigeon brain in the presence of pyruvate (0.027 *M*) and fumarate (0.005 *M*)*

1.5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, O₂; 38°.

Time min.	μl. O ₂ uptake (average of duplicates)			Extra O ₂ uptake caused by	
	No addition	10 μg. Vitamin B ₁	1.3 μg. cocarboxylase	Vitamin B ₁	Cocarboxylase
10	119	126	174	7	52
20	190	207	288	17	114
30	258	291	401	33	189
40	314	354	476	40	192
50	362	396	522	34	138

Even the smallest amount of cocarboxylase used in the experiments of Table VII (cf. specially Exp. 6), 0.15 $\mu\text{g.}$ for 140 mg. tissue (equivalent to 1.07 $\mu\text{g.}$ for 1 g. tissue), produced practically maximum effects; hence the average maximum catatorulin effect may be calculated for the first six experiments of Table VII with avitaminous brain. The value is 55 $\mu\text{l. O}_2$ in 30 min. or 580 $\mu\text{l. O}_2/\text{g. tissue} \times \text{hr.}$, which is about the same value as is reached with maximum amounts of vitamin B₁ in brei for the period 1–2 hr. In the dispersions the initial values for the first 10 min. are usually much higher; thus in Exp. 6 the effect reached the phenomenal increase of 1500 $\mu\text{l. O}_2/\text{g. tissue} \times \text{hr.}$ This is a larger increase than has ever been recorded in a catatorulin test with brei and yet it was produced, in this case, by 0.15 $\mu\text{g.}$ cocarboxylase equivalent to approx. 0.1 $\mu\text{g.}$ vitamin B₁; the latter would not produce a maximum rise of more than 100 $\mu\text{l. O}_2/\text{g.} \times \text{hr.}$ with the same amount of brei.

In dispersions of type I vitamin B₁ is inactive (some activity however occurred in Exp. 4, Table VII, with 20 $\mu\text{g.}$); as there is some cocarboxylase present in the avitaminous brain this shows that there is no stimulation by vitamin B₁ of the action of cocarboxylase similar to that found by Ochoa [1938] for yeast. Moreover, addition of vitamin B₁ together with cocarboxylase (Table VII) does not increase the effect of the latter alone.

Table IX. *Comparison of the effects of vitamin B₁ monophosphate and cocarboxylase on the O₂ uptake of dispersions (type II) from avitaminous pigeon brain, in the presence of pyruvate (0.027 M) and fumarate (0.005 M)*

1.5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, air; 38°. (Figures in brackets to the right give the extra O₂ taken up in the presence of catalyst.)

	$\mu\text{l. O}_2$ uptake in 30 min. (average of duplicates)	
	Exp. 1	Exp. 2
No addition	473	364
Vitamin B ₁	(0.5 $\mu\text{g.}$) 474 (-)	(1.0 $\mu\text{g.}$) 390 (+ 26)
Vitamin B ₁ monophosphate	(0.3 $\mu\text{g.}$) 469 (-)	(0.6 $\mu\text{g.}$) 388 (+ 24)*
Cocarboxylase	(0.01 $\mu\text{g.}$) 484 (+ 11)	(0.025 $\mu\text{g.}$) 398 (+ 34)
		(0.3 $\mu\text{g.}$) 463 (+ 99)

* 0.6 $\mu\text{g.}$ vitamin B₁ monophosphate contained 0.012 mg. cocarboxylase.

As with brei, there is no action upon dispersions from the brain of the "rice-fed" bird (Exp. 7, Table VII) not yet showing symptoms. Hence it seems clear that the 1.2 $\mu\text{g./g.}$ cocarboxylase, found in the brain of the "rice-fed" pigeon, are sufficient to supply the full needs of the enzyme system and that the extra 2 $\mu\text{g./g.}$ in normal brain [Ochoa & Peters, 1938, 1] constitutes a reserve. Since a decrease of 0.8 $\mu\text{g./g.}$ is associated with the appearance of symptoms (0.4 $\mu\text{g./g.}$ cocarboxylase in avitaminous brain) if cocarboxylase is the active form it would follow that for 140 mg. avitaminous tissue a maximum effect should be produced in the bottle by 0.11 $\mu\text{g.}$ This is reasonably close to the amount giving maximum action in Exp. 6 (Table VII).

The effects of increasing concentrations of cocarboxylase below the maximum are shown in Fig. 3 for a dispersion of type II. Amounts as low as 0.01 $\mu\text{g.}$ have a definite effect. 0.3 $\mu\text{g.}$ (with 320 mg. tissue) gives practically maximum activation, no increase occurring by using 1.3 $\mu\text{g.}$ 0.3 $\mu\text{g.}$ cocarboxylase in 320 mg. brain would correspond to about 0.9 $\mu\text{g./g.}$ tissue; this amount is again remarkably close to the 0.8 $\mu\text{g./g.}$ required to raise the cocarboxylase content of the avitaminous to that of the "rice-fed" brain.

Vitamin B₁ monophosphate is not more active than is vitamin B₁ itself in dispersions from avitaminous brain. Table IX shows the results of two experiments with dispersions of type II. The synthetic specimen of vitamin B₁ monophosphate used was free from vitamin B₁, but contained 2% cocarboxylase as tested with yeast.

C. *Synthesis of cocarboxylase by brain tissue in vitro*

The effect of vitamin B₁ in dispersions of type II, as well as in brei, is due to its enzymic phosphorylation to cocarboxylase. Although this only occurs to a very small extent, it is fully sufficient to account for the observed catatorulin effects. At the end of the experiments given in Table VIII and Fig. 2 cocarboxylase was determined (in quadruplicate) in the control samples as well as in those which had received vitamin B₁. The results are shown in Table X. In

Table X. *Synthesis of cocarboxylase from vitamin B₁ in dispersions (type II) from avitaminous pigeon brain (experiments of Table VIII and Fig. 2)*

Cocarboxylase determined at the end of experiments in samples with no addition and vitamin B₁ addition.

Exp.	Duration min.	μg. cocarboxylase per sample (320 mg. brain)		
		In control sample	In vitamin B ₁ sample	Synthesis
Table VIII	50	0.26	0.30	0.04
Fig. 2	40	0.16	0.21	0.05

320 mg. brain tissue (being the approximate equivalent of each sample) about 0.05 μg. cocarboxylase is synthesized. In a fresh experiment therefore the activities of 10 μg. vitamin B₁ and 0.05 μg. cocarboxylase were compared. Table XI shows that they produced almost the same O₂ uptake. But whereas

Table XI. *Comparison of the effects of vitamin B₁ and cocarboxylase, in dispersions (type II) from avitaminous pigeon brain, on the O₂ uptake in the presence of pyruvate (0.027 M) and fumarate (0.005 M)*

(A) 1.5 ml. enzyme (about 320 mg. brain) to 2 ml. with additions. Gas, O₂; 38°.

		μl. O ₂ uptake (average of duplicates)			Extra O ₂ uptake caused by		
Time min.	No addition	10 μg. vitamin B ₁	0.05 μg. cocar- boxylase	0.1 μg. cocar- boxylase	10 μg. vitamin B ₁	0.05 μg. cocar- boxylase	0.1 μg. cocar- boxylase
10	148	158	180	200	10	32	52
20	256	297	310	344	41	54	88
30	327	385	400	452	58	73	125
40	385	487	465	525	140	80	140

(B) Rates of extra O₂ uptake (in μl./hr.) caused by

Time period min.	10 μg. vitamin B ₁	0.05 μg. cocarboxylase	0.1 μg. cocarboxylase
0-10	60	190	310
10-20	186	130	216
20-30	100	114	222

the effect due to a given amount of cocarboxylase is practically maximum at the end of the first 10 min. and then decreases, the effect of the free vitamin is not maximum at the end of that time; it increases for the next 10-20 min. This, while adding further support to the view that the effect of the vitamin is due to

its progressive phosphorylation to cocarboxylase, makes a strict comparison of the vitamin and cocarboxylase effects difficult. Still, the agreement between the catatorulin effects of both in Table XI is reasonably good especially at the end of 20–30 min. Unpublished experiments of one of us (S. O.) show that at 38° the amount of cocarboxylase synthesized *in vitro* by various tissues from vitamin B₁ does not increase much after 30 min.

It remains to be shown that the brei used in the ordinary catatorulin tests will synthesize sufficient cocarboxylase to produce the observed action. A previous estimate of this synthesis by one of us [Peters, 1937] was in error owing to lack of knowledge of the stimulant action of vitamin B₁ itself in yeast. The experiments of Table XII show that with brei too there is small but sufficient

Table XII. *Synthesis of cocarboxylase from vitamin B₁ under the conditions of a typical catatorulin test with brei from avitaminous pigeon brain*

Each bottle contained an average of 140 mg. tissue in 3 ml. Ringer-phosphate pH 7.3. Gas, O₂; 38°. Pyruvate 0.018 M.

No.	Time min.	$\mu\text{l. O}_2$ uptake/g. tissue (average of duplicates)		
		No addition	2 $\mu\text{g.}$ vitamin B ₁	Extra due to vitamin
1	10	377	499	122
	20	682	850	168
	30	946	1270	324
2	10	326	363	37
	20	655	786	131
	30	878	1172	294
$\mu\text{g. cocarboxylase/g. tissue}$ (determined at end)				
1	—	0.63	0.82	0.19
2	—	0.60	0.70	0.10

Average cocarboxylase synthesis about 0.15 $\mu\text{g./g.}$ brain or 0.02 $\mu\text{g.}$ in an actual sample (140 mg. tissue).

synthesis. The average catatorulin effect at the end of 30 min., in the two experiments of Table XI, was 618 $\mu\text{l. O}_2/\text{g.} \times \text{hr.}$ and the cocarboxylase synthesized 0.15 $\mu\text{g./g.}$ Reference to the curve of Fig. 3 will show that 0.15 $\mu\text{g.}$ cocarboxylase produces in dispersions of type II in 30 min. an approximate extra O₂ uptake of 125 $\mu\text{l.}$ for 320 mg. tissue. This corresponds to 780 $\mu\text{l./g.}$ tissue $\times \text{hr.}$ a figure very close indeed to the value obtained with brei in Table XII. Hence the agreement is complete that oxidation of pyruvate here is catalysed by the cocarboxylase synthesized from the added vitamin B₁.

DISCUSSION

C₄ dicarboxylic acids. The effect of fumarate is a striking confirmation of views advanced in Szegeed of the fundamental importance of the system of C₄ dicarboxylic acids. Szent-Györgyi and his colleagues have suggested that the catalytic activity of these acids involves the two systems succinic-fumaric and malic-oxaloacetic acids. According to them H from the substrates (donators) is transferred to oxaloacetate which is thus reduced to malate; from malate H is transferred to fumarate which is reduced to succinate and this, activated on the succino-dehydrogenase, gives up its H to the cytochrome system. This explains why all these acids are effective in catalysis. It would also give a biological reason for the presence in brain tissue [cf. especially Weil-Malherbe, 1937] of a system capable of forming succinic acid from pyruvic acid, because

this would ensure the formation (from pyruvic acid) of the C_4 acids necessary for catalysis. Such an explanation would make it unnecessary to believe that the formation of C_4 acids from C_3 acids means that the pyruvate is oxidized through the stages of C_4 acids, a view which from several angles has been shown to be untenable [cf. Long *et al.* 1939].

Reaction in which cocarboxylase is involved. This paper adds final evidence to the view that cocarboxylase is the active form of vitamin B_1 concerned in the oxidation of pyruvate by animal tissues. The analogy with yeast and all the evidence derived from work with bacteria [Lipmann, 1937; Hills, 1938; Barron & Lyman, 1939] suggest that cocarboxylase is involved in a stage of (oxidative) decarboxylation of pyruvic acid. Recent work from this laboratory [Long & Peters, 1939] on the metabolism of α -keto-acids (and especially α -ketobutyric acid) in brain supports this view, since the extra O_2 uptake caused by the α -keto-acid in avitaminous brain brei is stimulated by vitamin B_1 and oxidative decarboxylation is the only metabolic change that this α -keto-acid undergoes in brain.

Catalytic activity of cocarboxylase. The catalytic activity of cocarboxylase with pyruvate in our brain dispersions is of a very high order. Assuming $0.01 \mu\text{g.}$ to be the smallest effective amount under our experimental conditions, this corresponds to a minimum effective concentration of $5 \times 10^{-9} M$. With $1.5 \times 10^{-7} M$ maximum effects are obtained. It can be calculated that, under optimum conditions, 1 mol. cocarboxylase catalyses the uptake of 1500 mol. O_2 per min. This is the same order of activity as found by Warburg & Christian [1938] for the coenzyme of the *d*-amino-acid oxidase. Also the activity of cocarboxylase in yeast, in the presence of an excess of vitamin B_1 is of the same order [cf. Ochoa & Peters, 1938, 1], but only about 1/10 of that in the absence of vitamin.

The brain enzyme is saturated with about $1 \mu\text{g.}$ cocarboxylase per 1 g. fresh brain. In yeast it can be calculated from data from various sources [i.e. Lohmann & Schuster, 1937; Ochoa & Peters, 1938, 1] that 1 g. fresh yeast is saturated with approximately $90 \mu\text{g.}$ cocarboxylase in the absence of free vitamin B_1 , or with $9 \mu\text{g.}$ in the presence of the latter. In any case the concentration of pyruvate dehydrogenase in brain seems to be much lower than that of carboxylase in yeast.

Vitamin B_1 metabolism. Now that the nature of the vitamin B_1 component of the pyruvate oxidation in animal tissues appears to be settled, it will be possible to consider with greater confidence the metabolism of the vitamin in a wider sense. The difficulty of penetration of the tissue brei by the phosphorylated form which is here shown, supports the idea that vitamin B_1 permeates the tissue walls as such, and is then phosphorylated within the cell. This is consistent with recent findings of Sinclair [1939] on the absence of cocarboxylase (and presence of vitamin B_1) in cerebrospinal fluid, as well as with the estimations of cocarboxylase in blood showing its presence in cells and its absence from plasma [Goodhart & Sinclair, 1939]. The physiological significance of the high phosphorylating powers for vitamin B_1 shown by liver and kidney is probably that these organs act as traps and store the vitamin, supplying it in the free form to the tissues according to their needs; both these organs show a high phosphatase activity towards cocarboxylase [Ochoa, unpublished].

It is difficult to understand why no catatorulin effects have ever been obtained with the avitaminous liver. Obviously the cocarboxylase in liver must also be playing the usual part in the oxidation of pyruvate. It is possible that it might also be concerned there with fat synthesis. McHenry & Gavin

[1938] have shown that administration of vitamin B₁ leads to storage of fat from carbohydrate; this effect is probably secondary to the oxidative decarboxylation of pyruvic acid.

Although it is shown in this paper that vitamin B₁ pyrophosphate is the active form concerned with oxidation of pyruvate, and free vitamin B₁ (or its monophosphate) occurs only in small amounts in tissues, it appears that vitamin B₁ in combination with protein is present in some biological fluids (plasma [Goodhart & Sinclair, 1939]; milk [Houston & Kon, 1939]). Whether this might be a transport form of the vitamin, or whether it has a different biological significance, it is impossible at present to decide.

SUMMARY

1. The system of C₄ dicarboxylic acids of Szent-Györgyi *et al.* is catalytically concerned in the oxidation of pyruvate in brain.

2. With brain slices and brei (avitaminous pigeon) cocarboxylase is less active in the cataturulin test than free vitamin B₁.

3. With two types of finely ground preparations of avitaminous brain, cocarboxylase is much more active than free vitamin B₁, one of the preparations reacts to cocarboxylase and not at all to vitamin B₁. Vitamin B₁ monophosphate is not more active here than is vitamin B₁ itself.

4. Brain preparations (including brei) which respond to vitamin B₁ can be shown to synthesize cocarboxylase in amounts which account for the changes in O₂ uptake found.

5. A maximum response is produced with a concentration of 1.5×10^{-7} M cocarboxylase; each mol. cocarboxylase catalyses optimally the uptake of 1500 mol. O₂ per min.

6. The hypothesis of Lohmann & Schuster that the active form of vitamin B₁ in animal tissues is the pyrophosphate is considered to be now proved.

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CXXXVI. THE ELECTROPHORETIC ANALYSIS OF NORMAL HUMAN SERUM

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It has been shown by Stenhagen [1938], using the Tiselius [1937, 1] electrophoresis technique, that normal human serum contains four electrophoretically distinct protein components, viz. albumin, and α , β and γ globulins. In this respect it is similar to the normal serum of other animals, e.g. horse, rabbit [Tiselius, 1937, 2].

The optical method used by Stenhagen does not allow of the accurate estimation of the relative quantities of the protein components of normal human serum. Such information is important, particularly for the proper interpretation of the changes which can be observed in sera of pathological human conditions.

This paper is an account of the investigation of several normal human sera, in which the electrophoretic behaviour has been observed by means of the Lamm [1928; 1929; 1937] scale method. It has thus been possible to make accurate estimates of the relative amounts of the protein components present.

EXPERIMENTAL

(1) *Treatment of sera*

The sera, obtained from normal fasting adults, were dialysed at constant volume in cellophane tubes against suitable buffers of ionic strength $\mu=0.1$, in a bath at 2° . The volume of buffer for dialysis was 5–10 times that of the serum sample. The dialysate was changed twice a day until ionic equilibrium was attained. Refractive index measurements ($\lambda=546\text{ m}\mu$) were made with the dipping refractometer on fresh buffer, and on buffer which had been in contact with the serum for 12 hr. When no difference in refractive index could be observed between these samples, it was assumed that equilibrium had been attained. The process usually took 3–4 days.

The dialysed serum was centrifuged to remove the slight precipitate which invariably formed, and the refractive index measured. For electrophoretic examination the serum was then diluted with buffer until the difference in refractive index between the protein solution and the buffer, $(n_1 - n_0)$, was 0.00300, where n_1 =refractive index of protein solution and n_0 =refractive index of buffer. This involved a fourfold dilution approximately.

The electrophoresis was carried out in a bath at 0° the potential gradient applied usually being about 5 V./cm.

(2) *Optical methods*

The electrophoretic migration was observed both by the Toepler "Schlieren" method [Tiselius *et al.* 1937] and by the Lamm scale method. A sodium vapour lamp was used as a light source and photographs taken on Ilford Half Tone Panchromatic Plates.

In the scale method a double equal distance scale was photographed through the U-tube, one scale through each limb. The scale was situated approximately 4.0 cm. from the centre of the U-tube. Immediately after the formation of the boundary in any experiment, the boundary being at that time hidden by the centre plate of the U-tube, an exposure was made which was used as a reference scale. The boundary between buffer and diluted serum was then brought into view by the compensator, the current switched on, and exposures taken subsequently at suitable intervals, usually every 30 min. "Schlieren" photographs, taken immediately before each scale photograph, were used for the selection of the most suitable scale photographs for measurement and plotting.

The electrophoresis curves were obtained by plotting the deviations of the scale lines, Z , against the deviated scale line position, X , in the cell, in accordance with the accepted method.

(3) *Estimation of amounts of components*

The type of curve obtained from the scale photographs shows some variation, depending on whether the electrophoretic migration takes place in the direction of the buffer or of the diluted serum. Greater spreading of the individual boundaries occurs in that limb of the U-tube in which the direction of migration is towards the diluted serum, and diagrams from this limb are therefore less suitable for the estimation of the amounts of all the components present.

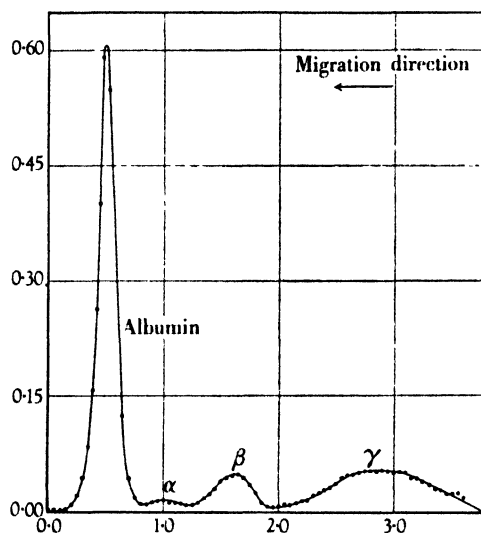


Fig. 1. Normal human serum. 3. Electrophoretic analysis. Phosphate buffer $pH\ 8\ \mu=0.1$ 4.9 V/cm. Exposure 152 min. after starting current. Anode limb. Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm.

In Fig. 1 is shown a typical scale diagram of a normal human serum, from that limb of the U-tube in which migration was taking place in the direction of the buffer layer. It is obvious that by slight extrapolations to the base line it is possible to measure the area enclosed by each peak, and this is proportional to the refractive increment due to each component [McFarlane, 1935, 1].

It can be seen from Fig. 2, a scale diagram for the opposite limb (migration towards diluted serum), that in this case the extrapolations are more uncertain. Such curves have been used for the estimation of the relative amounts of albumin and total globulin, but not for the estimation of the individual globulins.

The amounts of the components are given as percentages of the total refractive increment due to the protein present. It is not possible to express these values in terms of g. protein per 100 ml. serum as the specific refractive increments of the components probably differ, and reliable values for human serum proteins are not yet available.

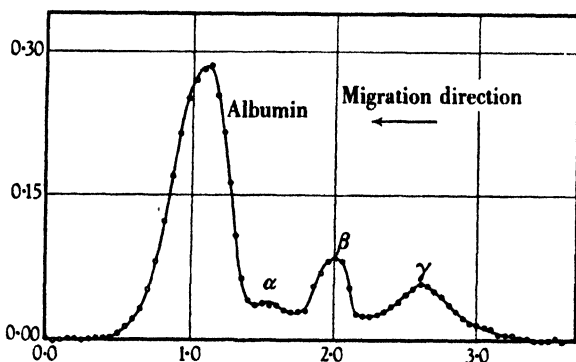


Fig. 2.



Fig. 3.

Fig. 2. Normal human serum. 3. Electrophoretic analysis. Phosphate buffer pH 8, $\mu=0.1$, 4.9 V./cm. Exposure 124 min. after starting current. Cathode limb. Abscissa: distance in U-tube in cm. Ordinate: scale line displacement in mm.

Fig. 3. Normal human serum. 5. Ultracentrifugal analysis. Phosphate buffer pH 8, $\mu=0.1$. $n_1 - n_0 = 0.00300$. Interval between exposures 10 min. Field strength 270,000 \times gravity. (1) Cell index, (2) globulin, (3) albumin, (4) meniscus.

(4) Ultracentrifuge technique

Samples of the solutions used for the electrophoresis experiments were examined in the ultracentrifuge in a field strength of 270,000 times gravity. The "diagonal schlieren" method [Philpot, 1938] was used for the observation of the sedimentation, a high pressure mercury arc being used as a light source. Photographs were taken on Ilford Half Tone Panchromatic Plates, monochromatic light $\lambda = 546 \text{ m}\mu$, being isolated by a suitable filter.

Fig. 3 is a series of photographs obtained by this method, showing the sedimentation behaviour characteristic of normal human serum. For the estimation of the amounts of the components, the centrifuge photographs were projected, with a magnification of six diameters, on to transparent graph paper. The contours of the enlarged image were traced freehand on the graph paper and the curves so obtained analysed geometrically. An arbitrary straight base line was drawn connecting those portions of the actual base line visible at the

extremities of the diffusion boundary. A symmetrical curve was constructed for the albumin peak and the remaining area assigned to globulin.

That the use of such an arbitrary base line was justified under the experimental conditions used, was shown by experiment. Following a normal serum sedimentation run, buffer alone was put into the cell and exposures taken at times corresponding to those in the serum run, with identical optical settings. By this means a true base line for each exposure was obtained. An analysis for albumin and globulin using the true and arbitrary base line gave the following figures: true base line, albumin 79 %, globulin 21 %; arbitrary base line, albumin 81 %, globulin 19 %.

Using either the arbitrary or true base line, the curve for the globulin component obtained by the geometrical analysis showed slight deviations from a perfectly symmetrical appearance. These deviations, representing about 2 % of the total curve area may have been due to the inclusion of some of the X fraction [McFarlane, 1935, 2] with the globulin. The quality of the curves, however, did not seem to justify a finer analysis.

RESULTS

The area enclosed by curves obtained by the Lamm scale method at a diffusion boundary such as is under consideration is given by the equation

$$A = Gab (n_1 - n_0),$$

where

A = area.

G = photographic enlargement factor.

a = thickness of fluid column.

b = optical distance from the scale to the middle of the cell.

n_1 = refractive index of the protein solution.

n_0 = refractive index of the buffer in which the protein is dissolved.

Substituting the values $G=0.75$, $a=2.5$ cm., $b=3.0$ cm. (corrected for the refractive index of water etc.), and allowing for a plotting magnification of 5×10^3 , a value of 84 cm.² is obtained for A . Comparison with the data of Table I shows that the areas found experimentally are in fair agreement with this value. The fact that the anode curve areas are generally slightly greater than those for the cathode will be referred to in the discussion.

Table I. *Comparison of anode and cathode curve areas*

Run no.	pH	Exposure no.	Curve areas	
			Anode	Cathode
12	8.0	7	74.8	71.3
13	8.0	6	79.4	76.7
14	8.0	6	79.9	77.5
15	8.0	7	80.7	78.9
16	7.0	6	75.4	78.3
17	4.0	6	71.9	71.2

The curve areas are in cm.²

In Table II, the relative quantities of albumin and total globulin, calculated from corresponding anode and cathode limb exposures are presented for a number of sera. The amount of albumin calculated from the cathode limb curve is in each case somewhat higher than that from the anode limb, and may be related to the area discrepancy noted above.

Table II. *Percentages of albumin and total globulin calculated from corresponding anode and cathode limb exposures*

Serum no.	pH	Exp. no.	Anode limb		Cathode limb	
			Albumin	Globulin	Albumin	Globulin
3	8.0	6	60.0	40.1	66.1	33.9
4	8.0	6	60.7	39.3	65.8	34.2
5	8.0	6	56.6	43.4	62.0	38.0
6	8.0	7	54.6	45.4	57.9	42.1
6	7.0	6	58.5	41.4	60.3	39.9
Mean			58.0	42.0	62.5	37.5

Mean values given to 0.5%.

Values are percentages of total refractive increment.

It has been pointed out by Tiselius [1937, 1] that it is usually most convenient to separate the protein components of sera at pH 8. This pH has also been found most useful for the present experiments. There remains the possibility, however, that the amounts of the components might vary with pH, and also, in view of the results of McFarlane [1935, 1, 2] with the ultracentrifuge, the amounts of the components might be expected to vary with the total protein concentration. These points have been investigated and the relevant data are given in Table III. The amounts of the components represent the mean values obtained from two successive exposures taken 30 min. apart with the current running continuously. The values refer to the limb in which migration was taking place towards the buffer; in most cases this was the anode limb. These conditions apply also to Table IV.

Table III. *Percentages of components at various pH and concentrations*

pH	$n_1 - n_0$	Albumin	Globulins		
			α	β	γ
8.0	0.00300	57.1	3.0	13.5	26.5
8.0	0.00150	57.2	4.7	13.0	25.3
7.0	0.00300	58.9	3.7	13.1	24.5
4.0	0.00300		79.4		20.6

Data obtained from normal human serum 6.

Values are percentages of total refractive increment.

Table IV. *Percentages of components in various normal human sera. pH 8*

Serum no.	Albumin	Globulins		
		α	β	γ
1	58.9	4.4	11.0	25.7
3	59.4	2.8	10.0	27.9
4	62.4	5.2	9.9	22.6
5	57.8	6.6	11.7	24.0
6	57.1	3.0	13.5	26.5
Mean	59.0	4.5	11.0	25.5

Analysis at pH 8. $n_1 - n_0 = 0.00300$.

Mean values given to 0.5%.

Values are percentages of total refractive increment.

Samples of a single normal human serum were dialysed as described against buffers of ionic strength $\mu = 0.1$ of pH 4, 7 and 8 and an electrophoretic analysis

made at the standard protein concentration. In addition the sample at pH 8 was investigated at half the standard concentration, viz.:

$$n_1 - n_0 = 0.00150.$$

It will be noticed that the values for the amounts of the components determined at pH 7 and 8 are in excellent agreement. At pH 4, even after prolonged electrophoresis, it was possible only to estimate the amount of γ globulin, as there was insufficient separation between the albumin, α and β globulin curves to allow any further analysis. The value obtained for the amount of γ globulin is in reasonable agreement with the values at pH 7 and 8.

Under the experimental conditions used there was no change in the amounts of the components with protein concentration. Owing to the design of the U-tube it was not possible to carry the investigations above a total protein concentration corresponding to $n_1 - n_0 = 0.00300$, as the scale deviations became so large as to be immeasurable, because of the out of focus effect.

In Table IV the results from the analysis at pH 8 of five normal human sera are assembled. It will be noticed that the relative quantities of the various components show a satisfactory constancy throughout the series. No attempt has been made to calculate standard deviations for the mean values given at the bottom of the table, owing to the rather small number of sera investigated.

Table V. *Comparison of electrophoretic and ultracentrifugal analysis of same sera*

Serum	pH	$n_1 - n_0$	Electrophoretic		Ultracentrifugal	
			Albumin	Globulin (total)	Albumin	Globulin
1	8.0	0.00300	58.9	41.1	80.0	20.0
3	8.0	0.00300	59.4	40.7	81.5	18.5
4	8.0	0.00300	62.4	37.7	80.0	20.0
5	8.0	0.00300	57.8	40.3	75.0	25.0
6	8.0	0.00300	57.1	43.0	76.0	24.0
6	8.0	0.00150	57.2	43.0	74.0	26.0
6	7.0	0.00300	58.9	41.3	78.5	21.5
6	4.0	0.00300	—	—	73.0	27.0

Values are percentages of total refractive increment.

No figures are included relating to the mobilities of the components in the various sera, as these would appear to be of doubtful significance when determined in the presence of other proteins, because the magnitude of the viscosity effects is unknown.

In Table V the ultracentrifugal analysis for each serum is compared with the corresponding electrophoretic analysis. The ultracentrifuge data represent the mean values from two exposures in each case; the electrophoretic data are the mean values from two anode limb curves.

The discrepancy between these two sets of results is marked and will be referred to in the discussion.

DISCUSSION

Referring to Table I, the question of the difference in the areas enclosed by the anode and cathode curves calls for some comment. The total area of the curves is very dependent on the accuracy with which the position of the base

line can be determined, and discrepancies of the same order as those shown in Table I frequently occur between areas from exposures on one side of the U-tube alone. The consistency with which the effect occurs, however, suggests that it may be significant, and this is supported to some extent by the data of Table II, for reasons that appear below.

Under the experimental conditions used in the work reported in this paper the only indication of a δ boundary [Tiselius, 1937, 2] has been a slight inflexion in the curve for γ globulin from a few of the exposures. Stenhagen [1938], with experimental conditions closely similar to those used here, reports the absence of a δ boundary. It has been suggested by Longsworth & MacInnes [1939] that the δ boundary is due to differences in buffer salt concentration between the protein solution and buffer, occurring on account of the Donnan equilibrium set up in the initial dialysis. It can be shown that dialysis at high protein concentration, followed by dilution with buffer, the technique used in these experiments, tends to minimize such differences. There has been no indication whatsoever of any ϵ boundary [Longsworth & MacInnes, 1939], from the cathode limb curves.

A δ boundary effect would tend to give a greater total area for the anode limb curves than for the corresponding cathode curves, and also tend to increase the relative amount of globulin as calculated from the anode limb, over that from the cathode limb curves.

The data of Tables I and II are consistent with this argument, though the effects are rather small. Further, the δ boundary effect would in these experiments be included in the γ globulin peak, resulting in too high a proportion for this component.

Tiselius & Kabat [1939], having excluded the δ boundary from their anode curves, found larger total areas for the cathode than for the anode curves, and report that "the occurrence of a δ boundary does not alter the relative concentration of the other components".

In view of the reservations of the previous paragraphs, the data of Table IV show satisfactory agreement, the amounts of the components being independent of pH and of concentration in the region examined (Table III). The significance of the marked discrepancy between the electrophoretic and ultracentrifugal analyses (Table V) is difficult to assess. The ultracentrifugal data are in general agreement with the results of McFarlane [1935, 1] and the protein concentration is such that any "mixture effect" is not detectable by further dilution. If the value for the amount of γ globulin is too high, as discussed above, this would be an aggravating factor, but it would seem that further analysis of the situation depends on a study of artificial mixtures of the electrophoretically separated components of normal human serum.

SUMMARY

1. The quantities of the protein components present in normal human serum have been determined by electrophoretic analysis.
2. Discrepancies between the electrophoretic and ultracentrifugal analyses are reported and discussed.
3. Electrophoretic analysis gives mean values of 59% albumin and 41% total globulin, the latter consisting of 4.5, 11.0 and 25.5% of α , β and γ globulin respectively.

By ultracentrifugal analysis the mean quantities of albumin and globulin found are 78 and 22% respectively.

4. The probable modifications in the electrophoretic analytical values consequent on a slight δ boundary effect, have been discussed.

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CXXXVII. QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN BIOLOGICAL MATERIAL

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(Received 11 May 1939)

A COLORIMETRIC method involving the use of cyanogen bromide and metol for the quantitative estimation of nicotinic acid was described in a previous paper [Bandier & Hald, 1939]. The practical value of the method was shown by a series of analyses on yeast. The method is very accurate and the colour has the merit of remaining constant for several days. The intensity of the colour is directly proportional to the concentration of nicotinic acid.

I. *The specificity of the method*

The method is based on the observation of König [1904] that a colour reaction ensues when pyridine reacts with CNBr and a primary or secondary aromatic amine.

If the analysis is carried out in the presence of KH_2PO_4 —as described below—no coloration ensues with small amounts of pyridine, nor will picolinic acid, α -picoline, trigonelline or methylpyridinium chloride cause coloration, even if heating with strong acid or alkali on a boiling water bath has first taken place. Larger amounts of pyridine (about 1 mg.) yield an inconstant and unstable pale yellow-red colour. Such a concentration of pyridine causes the solution to smell strongly of pyridine.

Like nicotinic acid, nicotinamide yields a clear yellow colour. By the present method of analysis, however, nicotinamide is hydrolysed to nicotinic acid; this happens in a few moments when it is heated with strong alkali or acid.

If KH_2PO_4 is not used, pyridine yields a deeper yellow-red colour which is also inconstant and unstable. Picoline gives a slight, almost negligible, coloration; the other compounds mentioned give none. The method thus appears to be fairly specific.

II. *Estimation of nicotinic acid in biological material*

The technique described in the above-mentioned paper for analysing dry yeast, is unsuitable for the determination of nicotinic acid in animal tissue (liver, muscle etc.). A method will now be described which on repeated trials has proved perfectly satisfactory for the estimation of nicotinic acid in animal tissues.

(1) *Technique.* The sample of tissue is minced, dried (in a dry current of air heated to 40°) and coarsely ground. The loss of weight is calculated. 5 ml. 4*N* NaOH are run into a 10 ml. graduated flask with a glass stopper and 2.5 g. dried material are placed in the liquid with a glass spatula. The flask is closed with a wad of non-absorbent cotton-wool and placed in a boiling water bath for about 30 min., during which time it is shaken once or twice. This causes complete solution of the organic material. The mixture is cooled a little (not too much, as gelatinization then takes place) and 1 ml. conc. 36% HCl is added;

the stopper is tightly fitted and the flask turned upside down twice. After cooling for a moment, a further 0.8 ml. conc. 36 % HCl is added and the contents are shaken as before. As there is considerable formation of gas precautions must be taken to prevent the stopper from being blown out. After cooling to 20° distilled water is added to make the volume up to 10 ml. After thorough shaking the mixture is centrifuged for about 10 min. in an ordinary centrifuge tube. An almost clear brownish fluid and a supernatant compact layer are obtained. 1 ml. of the liquid, representing 250 mg. dried organic material, is transferred to another tube and 9 ml. acetone are added. The tube is carefully closed with a rubber stopper; the stopper must be of a material that is not attacked by acetone, otherwise a milky turbidity appears on transference of the acetone extract to water. After vigorous shaking the mixture is centrifuged for 3–4 min. Two layers are formed: a small (about 0.3 ml.) highly coloured very viscid aqueous phase and a clear, slightly coloured, layer of acetone. 4 ml. of the acetone layer (corresponding to 100 mg. dried material) and about 3 ml. distilled water are run into a round-bottomed flask; the acetone is evaporated with a water vacuum pump without other heating than the warmth of the hand. The contents are then quantitatively transferred with the aid of 5 ml. 2 % KH_2PO_4 to a graduated flask of 20 ml. capacity and heated for 5 min. in a water bath at 75–80°. 1 ml. 4 % freshly prepared aqueous CNBr is added and after standing 5 min. in the water bath, the flask is cooled under the tap to about room temperature. 10 ml. saturated (about 5 %) fresh aqueous metol are added and the volume is made up to 20 ml. with distilled water. After standing 1 hr. or more at room temperature protected from light, the colour developed is read off in a Pulfrich photometer (filter S. 43) with a blank (prepared simultaneously) containing the same amounts of CNBr, KH_2PO_4 , metol and distilled water to make the volume up to 20 ml.

The solution obtained by transference of the acetone extract to water is not quite colourless; the degree of coloration depends on the organ examined. Therefore, a second portion of 4 ml. of the acetone layer used for the analysis is taken and treated as above (transference to water + 5 ml. 2 % KH_2PO_4 —heating with CNBr), but instead of metol, distilled water is added to make up the volume to 20 ml. The colour is measured in the Pulfrich photometer (filter S. 43) with distilled water in the other cell and the value found is subtracted from the first reading. From this result and the coefficient of extinction determined in a standard solution (0.1 mg. nicotinic acid), the amount of the nicotinic acid in the dried or fresh organic material can be calculated.

(2) *Comments.* Added nicotinic acid is recovered quantitatively, which proves the reliability of the method. The time during which the material is subjected to hydrolysis is ample; periods varying from $\frac{1}{4}$ to 2 hr. have yielded identical results. If the hydrolysis is carried out first in alkali and then in acid, the result is the same as when alkaline hydrolysis only is used.

After heating the tissue with 5 ml. 4*N* NaOH and adding 1.8 ml. 36 % HCl the reaction is distinctly acid ($\text{pH} = 3\text{--}4$). Closer study has shown that a quantity of HCl equivalent to the amount of NaOH used is insufficient, i.e. a certain excess of HCl must be added. The presence of greater quantities of HCl (as much as 2 ml. 36 % HCl) has no influence on the result of the analysis. If KH_2PO_4 is omitted the analysis will give lower values. The amount used (5 ml. 2 % KH_2PO_4) has proved adequate. To obtain a correct reading in the Pulfrich photometer the extinction must not exceed 0.50; the size of the cell must, therefore, be chosen with this in view. The samples of tissue from various organs give blanks representing 5–25 % of the values of the actual analytical results.

The organic material is dried in order to minimize the volume of the reagents; the method may of course be adapted to direct analysis of fresh material.

(3) *Analysis of small amounts of material.* When only a very small amount is available, the analysis may be carried out as follows: 0.5 ml. 4*N* NaOH and 250 mg. dried organic material are placed in a graduated centrifuge tube. After closing the tube with a wad of non-absorbent cotton-wool the contents are heated on a boiling water bath for about $\frac{1}{2}$ hr. (the tube is shaken once or twice to ensure complete solution of the material). 0.18 ml. 36% HCl is added; after shaking and cooling distilled water is run in to make up the volume to 1 ml.; 9 ml. acetone are added and the analysis proceeds as before.

On transference of the acetone extract to water, a rather marked turbidity appears which disturbs the photometric reading; the former method involving the use of 2.5 g. dried substance has therefore been adopted as the normal procedure. Identical results can be obtained by the two methods.

(4) *Examples.* Pig kidney, wt. 189 g., dry wt. 37 g., i.e. dry wt. = 19.6% of wet wt. 2.5 g. dry wt., 5 ml. 4*N* NaOH and 1.8 ml. 36% HCl are used. 5 ml. 2% KH_2PO_4 are employed for the colour reaction.

Analysis: the extinction is 0.363 in 1 cm. layer.

mg./100 g.

Blank: extinction 0.215 in 5 cm. layer.

0.1 mg. nicotinic acid: extinction 0.444 in 0.5 cm. layer.

The extinction of nicotinic acid in 100 mg. dry renal tissue is therefore

$$0.363 - (0.215 \div 5) = 0.320.$$

This corresponds to a nicotinic acid concentration of	36.0
Calculated for fresh material this is	7.09

If 1.58 ml. 36% HCl (which are equivalent to the amount of NaOH) are used, the result is

With 2 ml. 36% HCl the result is	29.5
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i.e. the same as when 1.8 ml. are used.	35.5
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If KH_2PO_4 is omitted the result is	31.4
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With 1 ml. 2% KH_2PO_4 the result is	35.7
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With 2 ml. 2% KH_2PO_4 the result is	35.9
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With 5 ml. 2% KH_2PO_4 the result is	36.0
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If the same sample is subjected first to alkaline and then to acid hydrolysis the result is	36.3
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(1 g. dried organic material is heated on a boiling water bath with 2 ml. 4*N* NaOH and distilled water to make the volume up to 6 ml., then 1.35 ml. 36% HCl are added. After reheating for 20 min. on the boiling water bath, 2 ml. 4*N* NaOH are added, the mixture is cooled and the volume made up to 10 ml. by adding distilled water. The rest of the analysis proceeds as above.)

With the technique used for very small amounts of tissue (250 mg.) the result is	...	36.0
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Dried pig kidney. Found nicotinic acid mg./100 g.	Dried pig kidney + 40 mg./100 g. nicotinic acid. Total nicotinic acid found mg./100 g.	% added nicotinic acid recovered
36.0	77.0	102.5

(5) *Other results.* The results of analyses made on various organs and tissues are given in Table I. The organic material was obtained from recently slaughtered healthy animals; drying was effected within a few hours of the death of the animal. The figures give the mean values of duplicate analyses made on samples of two organs.

The amount of nicotinic acid found in pig and ox muscle is more than 200 times as great as the amount found by Karrer & Keller [1938]. This discrepancy is undoubtedly due to the procedure adopted by these investigators, i.e. evaporation to dryness of the tissue extract and extraction of the nicotinic

Table I. *The amount of nicotinic acid in some organs and tissues expressed in mg./100 g. of fresh material*

	Nicotinic acid, mg./100 g.		
	Pig	Ox	Cod
Liver	11.8	12.2	—
Kidney	6.83	Cortex 6.56 Medulla 5.17	—
Spleen	4.04	4.42	—
Heart muscle	5.34	5.93	—
Striped muscle	4.73	4.90	1.95
Testis	4.42	—	—
Ovary	3.84	—	—
Adrenal cortex	—	6.54	—
Adrenal medulla	—	4.90	—
Thyroid	1.59	3.00	—
Pancreas	5.00	—	—
Thymus	3.25	—	—
Roe	—	—	1.52

acid with warm benzene; this extraction is not quantitative—at any rate the method has failed in my hands.

The amount of nicotinic acid in pig and ox liver agrees well with the results of Swaminathan [1938] for sheep's liver.

III. *Estimation of nicotinic acid in some medicinal preparations*

The amount of nicotinic acid in some medicinal preparations (yeast, liver and stomach preparations) used in the treatment of pellagra is shown in Table II.

Table II. *The amount of nicotinic acid expressed in mg./100 g. or 100 ml. of some medicinal preparations*

Yeast preparations		Stomach preparations	
Name	Nicotinic acid mg./100 g.	Name	Nicotinic acid mg./100 g.
Levurine (A.B.)	47.8	Ventriculin (MCO)	16.9
Leogaer (Leo)	49.7	Ventriculin (P.D. and Co.)	13.2
Bevital (Leo)	47.7	Gastrovic (Leo)	15.4
		Ventriculus siccatus (Orthana)	15.0
		Intricula (Ido)	12.8
Liver preparations		For injection	
Name	Nicotinic acid mg./100 g.	Name	Nicotinic acid mg./100 g.
Exhepa (Ido)	122	Campolon (Bayer)	41.3
Mecolever (MCO)	119	Exhepa fort. p. inj. (Ido)	1.6
Exhepa liquid. (Ido)	43.7	Hepsol (MCO)	2.9
Extractum hepatis fluidum (A.B.)	6.0	Hepsol fortior (MCO)	116.0
Extractum hepatis fluidum (Exoglen)	32.3	Extract. hepatis p. inj. (Gea)	39.2
		Perhepar (Gedeon Richter A.G.)	49.4

The method described above has been used in analysing the dry preparations. As the fluid preparations contain fewer buffer substances, less HCl is needed after the alkaline hydrolysis to make the reaction slightly acid (pH about 4–5).

The preparations also have been analysed after the addition of a known amount of nicotinic acid; this has been recovered quantitatively in every instance.

Investigations by Spies *et al.* [1938], Vilter *et al.* [1939], Sebrell & Butler [1938], Chick *et al.* [1938], Day *et al.* [1938] and Langston *et al.* [1938] have shown, however, that nicotinic acid is not the only factor which plays a role in the therapy of pellagra. At present, therefore, it cannot be decided if the amount of nicotinic acid in organic preparations is the sole indicator of their therapeutic value in pellagra. There is no relation between the concentration of nicotinic acid in liver preparations and their therapeutic effect in pernicious anaemia.

With regard to considerations concerning the identity of the vitamin B₂ complex with the "cyanide-insensitive enzyme complex" composed of the "yellow respiration ferment" [Warburg & Christian, 1932], the coferment [Warburg & Christian, 1934] or cozymase [v. Euler *et al.* 1935] and the "Zwischenferment", the reader is referred to an article by the author which will shortly appear elsewhere.

SUMMARY

1. The colorimetric method for the quantitative estimation of nicotinic acid described in a previous paper is fairly specific, as small amounts of pyridine, picolinic acid, α -picoline, trigonelline or methylpyridinium chloride give no colour when the analysis is carried out in the presence of a certain amount of KH_2PO_4 .

2. The technique for analysing biological material and medicinal organic preparations is described.

3. The result is given of a series of analyses of pig and ox organs and tissues, as well as some organic preparations used in the treatment of pellagra.

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CXXXVIII. THE PHOSPHORIC ESTERS OF THE PANCREAS: SPHINGOSINE CHOLINE PHOSPHATE

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(Received 19 May 1939)

IN a former communication [King, 1932] it was shown that bone phosphatase would completely hydrolyse the phosphoric esters of muscle and of blood and that the optimum *pH* for the hydrolysis of esters from muscle was in the neighbourhood of the optimum for a phosphomonoesterase, while in the case of the esters from blood the optimum for hydrolysis corresponded more closely to that for the action of a diesterase. With complex mixtures of esters such as exist in tissue it is not reasonable to expect a well-defined *pH* optimum, but in the case of muscle the subsequent isolation by various workers of several phosphoric esters showed that most of them are optimally hydrolysed at an alkaline reaction. The isolation from blood of a large amount of diphosphoglyceric acid, an acid which is optimally hydrolysed near the neutrality point, is compatible with the *pH* hydrolysis curves of mixed esters from the blood.

In contrast with the phosphoric acid esters of blood and of muscle, those of some other organs have been found to be only partially hydrolysed by bone phosphatase at any *pH*. This is notably true in the case of pancreas tissue. It was found that only 40–50 % of the phosphorus of this organ is set free when treated with phosphatase even for very long periods. What this phosphoric ester (or esters) so refractory to the action of phosphatase, is, had not been determined. The present paper deals with the isolation of one of the esters.

EXPERIMENTAL

30 lb. of pigs' pancreas, packed in solid CO₂ about 10 min. after the killing of the animals, were treated with 7 % trichloroacetic acid and the extract fractionated as shown in Fig. 1.

The most striking feature of this fractionation is the very large amount of P which is not precipitable by basic lead acetate. Approximately 50 % of the organic P originally present in the trichloroacetic acid extract is not precipitated in the first three procedures. In the case of the phosphoric esters of muscle, blood and yeast fermentation, only very small amounts of P remain in solution after precipitation with basic lead acetate and ammonia [cf. Outhouse, 1935].

Phosphatase hydrolysis of fractions. A study of the phosphatase hydrolysis of the various fractions is shown in Fig. 2. The barium-insoluble fraction, like glycerophosphate, is practically completely hydrolysed at an alkaline reaction in 6 hr. The curve is not smooth, as in the case of glycerophosphoric acid. It shows an optimum *pH* compatible with the presence of primary esters, but there is also some indication of the presence of a secondary ester, or esters, or of some P compound similar to diphosphoglyceric acid.

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The phosphatase hydrolysis of the phosphoric acid esters from the mercury-insoluble and lead-insoluble fractions was similar to that of the barium-insoluble fraction: all esters were completely hydrolysed in 24 hr.

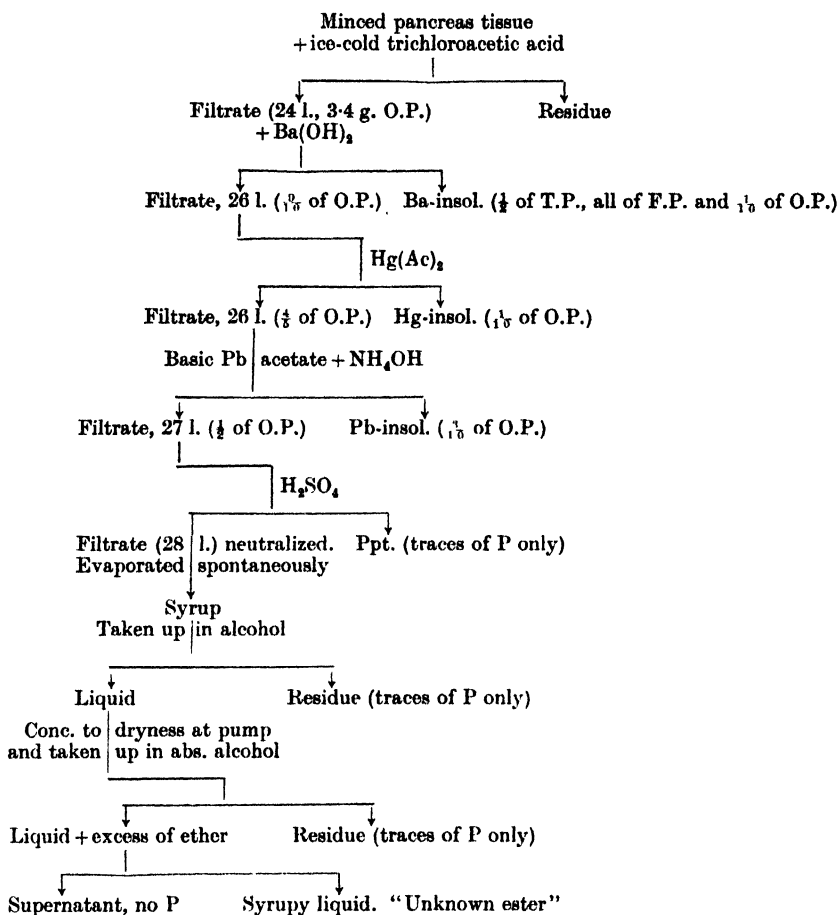


Fig. 1. Separation of phosphorus fractions from the pancreas. T.P. = total phosphorus, O.P. = organic phosphorus, F.P. = free (inorganic) phosphorus. All operations involving a departure from near neutrality in the reaction of the solution, carried out in the cold room.

In contrast is the almost complete absence of hydrolysis in the case of the lead-soluble fraction. It was thought that this phosphorus compound, so refractory to the action of phosphatase, might possibly be a secondary ester. If so, it should be hydrolysed by a secondary esterase. As Gulland [1938] has recently shown, snake venoms are very potent sources of phosphodiesterase. Solutions of the lead-soluble material were treated at different pH with a mixture of the venoms of *Crotalus oreganus* and *Naja Naja*, both rich sources of diesterase. Bone phosphatase was added to each. Considerable hydrolysis occurred during 24 hr. at pH values ranging from 7 to 9, the maximum hydrolysis amounting to a little over 60%.

Isolation of an ester from the lead-soluble fraction. The phosphorus-containing substance (or substances) is soluble in absolute alcohol, from which it may be

thrown down as a heavy oil or syrup with ether, ethyl acetate, acetone, light petroleum or chloroform. It is exceedingly soluble in water and because of its extremely hygroscopic nature it has been found difficult to obtain the

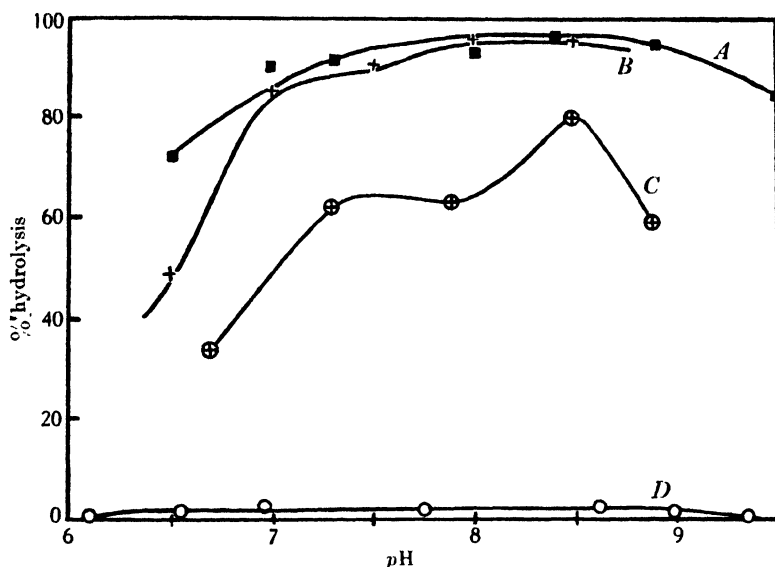


Fig. 2. Hydrolysis by bone phosphatase of phosphoric esters. A, glycerophosphate; B, esters precipitated by lead; C, esters precipitated by barium; D, "soluble ester". 6 hours at 37°.

substance in a condition satisfactory for analysis. Nevertheless, repeated precipitation with ether and with ethyl acetate and acetone, failed to change the P content to any appreciable extent.

In addition to P the material was found to contain N, half of which was in the amino-form. Repeated precipitations did not alter the P : N ratio. A very low reduction was given with the ferricyanide sugar reagent of Hagedorn and Jensen. Acid hydrolysis caused no increase in the reducing power. The iodine reduction (method of MacLeod & Robison [1929]) was much higher, probably owing to the presence of the amino group. There was no optical rotation. Comparative figures for several other phosphoric esters are shown in Table I.

Table I. *Comparison of soluble phosphoric ester from pancreas with other phosphoric esters*

Pancreas ester	Reducing power as glucose (%)		[α] _D	Hydrolysis constant $k \times 10^{-3}$
	H. and J.	Iodine		
Sphingosine choline phosphate	6	27	0	0.060
	"No reducing carbohydrate"		0	Nearly equal to glycerophosphate*
Aminohexahydric alcohol phosphate	2	2	0	Nearly equal to glucose phosphate†
Aminoethanol phosphate	—	—	—	0.067‡
Choline phosphate	—	—	—	0.23‡
Ba glucose-6-phosphate	35	45	21° [α] ₅₄₆₁ §	0.13§
Ba β -glycerophosphate	0	0	0	0.05

* Booth [1935].

† Calculated from data of Plimmer & Burch [1937].

‡ Outhouse & King [1935].

§ Robison & King [1931].

The properties of this very soluble P compound of the pancreas are in part similar to those of an ester from malignant tissue described by Outhouse [1933], and to the P-containing compounds shown to be present in embryonic tissue by Needham *et al.* [1937]; but its chief points of similarity seem to be with the water-soluble sphingomyelin-like substance of liver [Strack *et al.* 1933], and with the very soluble ester isolated from the kidney by Booth [1935] and thought to be the sphingosine phosphoric ester of choline. Booth isolated this ester by mercuric chloride precipitation from the alcoholic solution of the residue obtained on evaporation of the basic lead acetate filtrate. Precipitation of the present compound from alcoholic solution with mercury, followed by decomposition with H_2S in aqueous solution, evaporation and re-solution in alcohol did not alter the P or N content. Cadmium chloride likewise precipitates it from alcoholic solution; barium hydroxide gives no precipitate even when added to pH 12. The properties of the compound appear to be identical with those given by Booth for his ester.

The rate of hydrolysis for the phosphate group was determined in *N* HCl at 100°. It is difficultly hydrolysable like that of Booth's ester. The choline is split off much more easily than the phosphate group.

An examination of the products of prolonged hydrolysis with HCl showed the presence of choline, which was precipitated as the double salt with gold chloride. Its amount was estimated in the hydrolysate by the periodide method of Roman [1930], after heating a solution of the ester containing 2*N* HCl at 100° for 14 hr.

The analytical figures were as follows:

	P %	N %	Choline %
Found	6.40	5.80	25.5, 25.2
Calc. for sphingosine choline phosphate $C_{32}H_{51}O_6N_2P$	6.43	5.80	25.1

From the products of hydrolysis with hot barium hydroxide solution the barium salt of an ester was obtained which contained P and N in the ratio of 1 : 1. This is believed to be barium sphingosine phosphate. The amount of material finally available was insufficient for a satisfactory analysis.

The investigation of the esters contained in the barium- and lead-insoluble fractions is being continued, as well as the possibility of there being other esters in the lead-soluble fraction.

SUMMARY

Approximately one half of the acid-soluble phosphorus of the pancreas is unprecipitated by barium hydroxide, mercuric acetate and basic lead acetate. The ester-phosphorus of this very soluble fraction is hydrolysed with difficulty by acids and by bone phosphatase, but is hydrolysed by the combined action of phosphomonoesterase and phosphodiesterase. A substance, thought to be sphingosine choline phosphoric ester, has been isolated from this fraction.

Thanks are due to Mr C. E. Brett of Messrs T. Wall and Sons Ltd., for his kind co-operation in securing the pancreas glands for us.

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CXXXIX. THYROID AND VITAMIN B₁¹

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FROM 1930 onwards Abelin and colleagues have drawn attention to the importance of diet in general in experimental hyperthyroidism [e.g. Abelin, 1930]. In more specific work Himwich *et al.* [1932] claimed that in experimental hyperthyroidism dogs require more than the normal amount of vitamin B₁, and Cowgill & Palmieri [1933] found that pigeons with hyperthyroidism needed additional amounts of vitamin B₁ if their body weight was to be maintained. Later Sure & Buchanan [1937], using young rats, and Drill & Sherwood [1938], using more mature animals, were able to counteract with vitamin B₁ the fall in weight produced by thyroid administration.

During the course of an investigation on the tissue respiration of thyroid-treated rats, we soon became aware that the kidneys and livers were oedematous. This turned our attention to the relation between thyroid feeding and vitamin B₁. In experiments commenced before the last-mentioned paper appeared we have been able to confirm fully the results of the above workers. In addition Drill [1938], using a yeast fermentation method of estimation which has not been free from criticism, has reported a diminution of the vitamin B₁ content of several rat tissues following thyroid feeding. From catatorulin tests and determinations of the cocarboxylase content of boiled extracts from various tissues we can however state definitely that a genuine vitamin B₁ deficiency exists in the tissues of rats with hyperthyroidism.

Reagents used

Cocarboxylase. The cocarboxylase used as a standard was a synthetic product kindly presented by Messrs Merck (U.S.A.). This was standardized by comparison with a pure specimen of cocarboxylase from Dr K. Lohmann and found to contain 65 % of active material.

Vitamin B₁. Synthetic vitamin B₁ hydrochloride was kindly presented by Messrs Hoffmann La Roche and Messrs Bayer.

Sodium pyruvate. For the catatorulin experiments crystalline sodium pyruvate was prepared as previously described [Peters, 1938]. For the cocarboxylase experiments a preparation was made by Mr C. Long by the neutralization of pyruvic acid by sodium ethoxide in alcohol.

Desiccated thyroid and thyroxine. These were commercial products.

Baker's yeast. This was obtained from the Distillers Co., Ltd.

Our best thanks are due to Messrs Merck, Hoffmann La Roche and Bayer for their gifts.

¹ A preliminary report of these experiments has appeared in *J. Soc. chem. Ind., Lond.*, 58, 471, 1939.

² Harmsworth Senior Scholar, Merton College.

The rats used, all males, were taken from the usual laboratory stock. Throughout all the experiments the animals were fed on the following basal diet:

Rice starch	...	70
Casein	...	20
Salt mixture	...	5
Agar agar	...	2
Cod liver oil	...	3

To 100 g. of this diet were added 10 g. dry yeast.

The yeast and cod liver oil supplements provided the vitamin requirements. Mr H. W. Kinnersley has carried out curative pigeon tests and estimations by fluorescence of the vitamin B₁ in the yeast. He reports that after treatment with takadiastase the value obtained by the bird test (16 animals) was 4.8 µg./g., and by the thiochrome method 5.0 µg./g. He states (private communication) that, in the absence of takadiastase digestion, much less appeared to be present.

The average vitamin B₁ intake per day was about 7 µg. per animal. Experiments with over 40 animals have shown that there was no increase in growth rate if larger amounts of the vitamin were added to the diet. As far as vitamin B₁ is concerned the standard diet may be said to be adequate for normal growth and development.

(1) *Protection from loss of weight following thyroid administration by injection of vitamin B₁*

Three groups of 6 animals (av. wt. 180 ± 10 g.) were fed on the basal diet for 12 days. Group I was a control. Each animal in Group II was given daily 1 mg. thyroxine (dissolved in 0.5 ml. M/100 KOH) and 0.5 mg. synthetic vitamin B₁ hydrochloride (dissolved in 0.5 ml. 1% NaCl) by subcutaneous injection. Group III was given the thyroxine without the vitamin. It is seen in Fig. 1 that the animals in Group III (thyroxine only) rapidly lost weight, but that this fall was partially prevented if vitamin B₁ were given in addition (Group II). This completely confirms the results of Drill & Sherwood [1938].

This protection from fall in weight conferred by vitamin B₁ has also been shown in the following new experiment. Two further groups of animals (av. wt. 135 g.) were fed on the basal diet for 7 days, and at the end of this time a daily dose of 0.4 g. desiccated thyroid was given to each animal. During the 7 days on the basal diet only, Group V received daily by subcutaneous injection 250 µg. vitamin B₁ hydrochloride. Group IV received no such pretreatment. The pretreated animals (Fig. 2) fell in weight at a much slower rate than the untreated controls. Hence a pretreatment with vitamin B₁ also gives a protection against rapid fall in weight.

The weight changes in these feeding experiments are summarized in Table I. Application of Fisher's *t* test to the difference of means of Groups II and III gives *t*=4.33. For *P*=0.01, *t*=3.17. The difference between the means of Groups II and III is thus clearly significant.

The diet, although low in vitamin B₁, contains enough of the vitamin for normal growth as is evidenced by the control Group I. Apparently, however, it does not contain enough of the vitamin to give protection from the large falls in weight produced by thyroxine injections.

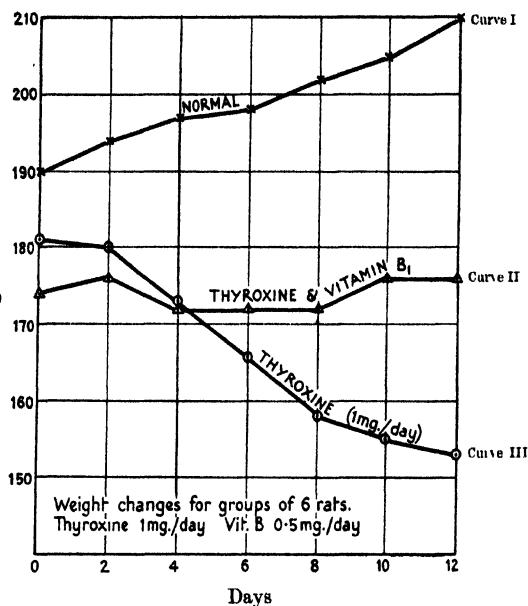


Fig. 1.

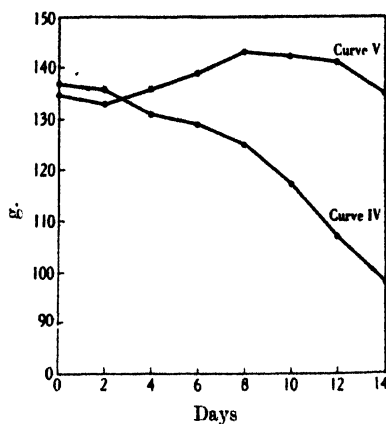


Fig. 2.

Fig. 1. Changes in weight of rats injected with thyroxine with and without vitamin B₁. Each curve = average for 6 rats. Curve I: control.* Curve II: thyroxine (1 mg. per day) plus vitamin B₁ (0.5 mg. per day). Curve III: thyroxine (1 mg. per day).

Fig. 2. Change in weight of rats fed on desiccated thyroid with and without pretreatment with vitamin B₁. Each curve = average for 8 rats. Curve IV: 0.4 g. desiccated thyroid per day (no pretreatment). Curve V: 0.4 g. desiccated thyroid per day (pretreated with 250 µg. vitamin B₁ per day for 7 days).

Table I. *Mean change in weight of rats*

Treatment	No. of animals	Mean change in weight (g.)	S.E.
I. Control	6	+ 20.2	± 2.6
II. Thyroxine (1 mg. per day). Vitamin B ₁ (0.5 mg. per day)	6	+ 2.2	± 2.1
III. Thyroxine (1 mg. per day)	6	- 26.6	± 7.8
IV. 0.4 g. desiccated thyroid per day (no pretreatment)	8	- 38.9	± 4.6
V. 0.4 g. desiccated thyroid per day (pretreated with 250 µg. vitamin B ₁ per day for 7 days)	6	- 1.0	± 4.1

(2) *Catatrolin effects with brain tissue*

The O₂ uptake of minced brain tissue taken from normal rats and rats with hyperthyroidism was determined, both in the presence and absence of added vitamin B₁, by the usual Warburg manometric technique, using sodium pyruvate as substrate. Each experiment was run for 2 hr. after a 15 min. equilibration period.

* The average weight of the control group (curve I) is some 10 g. higher than that of groups II and III. This was due to an oversight; we have no reason to believe that animals in a slightly lower weight group would react differently.

Table II shows the catatorulin tests, the % increase being calculated, as is the usual practice, for the period 60–120 min. [Peters, 1938]. Both rats fed on thyroid and those injected with thyroxine gave positive effects which were not observed with control animals on the same diet. Two thyroxine-injected animals which were treated for 12 days instead of the usual 16–18, however, did not show the effect.

Table II. *O₂ uptake (μl./g. fresh tissue/hr.) of normal and hyperthyroid brain tissue, with and without addition of vitamin B₁ (5 μg.). Substrate, sodium pyruvate. Temperature, 38°. The O₂ uptake was measured over the period 60–120 min.*

Exp. no.	Treatment	Days of treatment	Pyruvate	Pyruvate plus vitamin	Difference	% catatorulin effect
35	Normal	—	1733	1795	+ 62	+ 3.5
36	"	—	1682	1643	- 39	- 2.5
37	"	—	1644	1570	- 74	- 4.5
41	"	—	1377	1342	- 35	- 2
39	"	—	1515	1488	- 27	- 2
Mean (±s.e.) = -1.5 (±1.3)						
33	Thyroid-fed	16	1891	2115	+ 224	+ 12
34	"	16	1843	2111	+ 268	+ 14.5
38	"	15	1710	1806	+ 96	+ 5.5
56	"	17	1475	1648	+ 173	+ 12
57	"	17	1524	1684	+ 160	+ 10
61	"	18	1535	1565	+ 30	+ 2
45	Thyroxine-injected	18	1753	1911	+ 158	+ 9
58	"	18	1315	1429	+ 114	+ 8.5
59	"	18	1236	1360	+ 124	+ 10
60	"	18	1465	1638	+ 173	+ 12
Mean (±s.e.) = 9.6 (±1.1)						

Since O'Brien & Peters [1935] found that even with avitaminous rat cerebrum the catatorulin effects were small, the observed increases with hyperthyroid tissue are of interest.

Cohen & Gerard [1937], using the delayed substrate technique of Quastel & Wheatley [1932], find that for most substrates there is a higher O₂ uptake with hyperthyroid brain tissue than with normal. This does not apply to sodium pyruvate. Their technique however is not free from criticism, as small traces of substrate present may stabilize the enzyme systems [Peters, 1936]. Our observations indicate that with glucose brain tissue from hyperthyroid animals has a higher O₂ uptake than that from normal ones, and also confirm Cohen & Gerard's claim, qualitatively but not quantitatively. That the absence of effect found by them in the case of sodium pyruvate might be due to a vitamin B₁ deficiency is suggested by Table III. With this substrate, though there is no significant difference between the O₂ uptakes of normal and hyperthyroid tissue, with vitamin B₁ added the difference becomes significant.

Table III. *Average O₂ uptake (μl./g. fresh tissue/hr.) of brain tissue from normal and hyperthyroid rats, and of tissue from hyperthyroid rats after the in vitro addition of 5 μg. vitamin B₁. Substrate, sodium pyruvate. The O₂ uptake was measured over the period 60–120 min. Temperature, 38°.*

Treatment	No. of observations	Mean O ₂ uptake	S.E.
Normal	9	1580	±50
Hyperthyroid	12	1530	±65
Hyperthyroid + vitamin B ₁	14	1800	±72

(3) *Catatorulin effects with kidney tissue*

In early experiments Thompson [1934] reported catatorulin effects with kidney tissue from avitaminous animals, using sodium lactate. Table IV (sliced tissue) and Table V (minced tissue) show that with sodium pyruvate hyper-

Table IV. O_2 uptake ($\mu\text{l./g. fresh tissue/hr.}$) of normal and hyperthyroid kidney tissue (tissue slices) with and without the addition of vitamin B_1 ($5 \mu\text{g.}$). Substrate, sodium pyruvate. Temperature, 38° . The O_2 uptake was measured over period 60–120 min.

Exp. no.	Treatment	Pyruvate	Pyruvate plus vitamin	Difference	% catatorulin effect
16	Normal	3768	4979	+ 1210	+ 32
17	"	4257	5265	+ 1009	+ 24
18	"	3780	4842	+ 1062	+ 28
					Mean = + 28
15	Thyroid-fed	4050	4838	+ 784	+ 19
25	Thyroxine-injected	3818	5148	+ 1330	+ 35
					Mean = + 28
14	Thyroid-fed. Vitamin B_1 injected (1 mg. per day for 3 days)	4790	4565	- 225	- 4.5

Table V. O_2 uptake ($\mu\text{l./g. fresh tissue/hr.}$) of normal and hyperthyroid kidney tissue (tissue mince) with and without the addition of vitamin B_1 ($5 \mu\text{g.}$). Substrate, sodium pyruvate. Temperature, 38° . The O_2 uptake was measured over period 60–120 min.

Exp. no.	Treatment	Pyruvate	Pyruvate plus vitamin	Difference	% catatorulin effect
20	Normal	1725	2018	+ 293	+ 17
26	"	1640	1795	+ 155	+ 9.5
27	"	985	1238	+ 253	+ 26
					Mean = + 17
19	Thyroid-fed	2540	2857	+ 317	+ 12.5
22	Thyroxine-injected	1909	2100	+ 191	+ 10
23	"	1651	1853	+ 202	+ 12
24	"	1995	2380	+ 385	+ 19.5
25	"	2440	2933	+ 494	+ 20
					Mean = + 15
21	Thyroid-fed. Vitamin B_1 injected (1 mg. per day for 3 days)	2888	2785	- 103	- 3.5

thyroid kidney tissue gives large catatorulin effects; but tissue from normal animals gives effects of the same order which are abolished by previous daily injection of vitamin B_1 . It is of interest to note that the O_2 uptakes of kidney slices in pyruvate were very large during the first half hour period. Values as high as $8000 \mu\text{l./g. fresh tissue/hr.}$, representing a Q_{O_2} of 32, were recorded.

Catatorulin effects were also observed in both normal and hyperthyroid tissues with glucose, but they were never as large as with pyruvate. As usual, liver slices showed no vitamin effect in the presence of glucose or of butyrate, hexanoate or octanoate.

(4) *Coccarboxylase contents of boiled extracts of heart, kidney, liver and brain*

Method. The coccarboxylase contents of the boiled extracts of the various organs were determined by the method of Ochoa & Peters [1938]. The CO_2 produced by the enzymic decarboxylation of pyruvic acid in the presence of

excess free vitamin B₁ and Mg⁺⁺, was measured manometrically. Alkaline washed baker's yeast was used as a source of apocarboxylase and the CO₂ produced by a measured volume of boiled extract was compared with that produced by known amounts of the standard cocarboxylase.

The animals were killed by decapitation, and after bleeding the tissue was weighed in a weighing bottle, minced finely with forceps and scissors and ground in a small porcelain mortar with 2 vol. distilled water. The suspension was then plunged into a boiling water bath for 3–5 min., and after cooling was centrifuged; 0.3 ml. was used for each determination.

Each manometric vessel contained the following:

In main chamber

(1) 1 ml. yeast suspension (1 g. dry baker's yeast suspended in 10 ml. *M*/15 phosphate buffer pH 6.2. The yeast was washed 3 times with 45 ml. *M*/10 Na₂HPO₄ and once with 45 ml. distilled water.

(2) 0.1 ml. MgCl₂ solution (containing 10 µg. Mg).

(3) 0.1 ml. vitamin B₁ solution (containing 10 µg. vitamin B₁ hydrochloride).

(4) 0.3 ml. boiled extract or standard cocarboxylase solution.

In side chamber

0.2 ml. sodium pyruvate solution (90 mg. crystalline sodium pyruvate dissolved in 3 ml. *M*/15 phosphate buffer pH 6.2).

Nitrogen was used in the gas space but in some of the later experiments air was used. The difference was found to be negligible. The substrate was tipped after a preliminary 15 min. equilibration period, and the CO₂ evolved in 30 min. measured. The temperature was 28°.

Results

Animals not treated with vitamin B₁

Table VI gives the average value of the cocarboxylase in the boiled extracts from:

I. Animals fed on basal diet.

II. Animals fed on basal diet plus 0.4 g. desiccated thyroid per day.

III. Animals fed on basal diet but with the yeast supplement replaced by autoclaved marmite. This was a B₁-avitaminous diet and the animals were used when they showed typical symptoms of deficiency.

Table VI. *Average values for cocarboxylase contents of rat tissues (µg./g. fresh tissue)*

Group	Treatment	Tissue	No. of observations	Mean	2 × s.e. of mean
I	Basal diet	Heart	8	1.95	± 0.22
		Kidney	8	0.93	± 0.14
		Liver	7	1.32	± 0.28
		Brain	5	1.87	± 0.44
II	Basal diet plus 0.4 g. desiccated thyroid per day	Heart	8	1.31	± 0.24
		Kidney	8	0.63	± 0.16
		Liver	8	0.65	± 0.20
		Brain	5	1.39	± 0.40
III	Avitaminous diet (symptoms)	Heart	6	0.41	± 0.12
		Kidney	4	0.30	± 0.10
		Liver	6	0.43	± 0.08
		Brain	6	0.79	± 0.14

The individual values are given in the appendix (Tables IX, X and XI) and are calculated on the assumption that the tissues contain 70 % water. The

water content was found to vary from animal to animal, especially in the case of liver and kidney tissue. On the whole these tissues from hyperthyroid animals contained a slightly higher percentage of water than corresponding tissues from normal animals. But as the variation was never greater than 5%, and as dry weights were not determined in every case, the results were calculated on a wet weight basis on the above assumption.

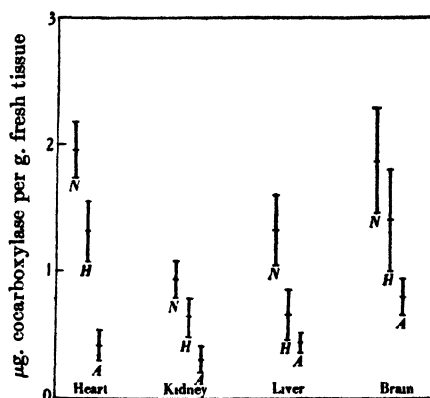


Fig. 3. Cocarboxylase ($\mu\text{g./g.}$ fresh tissue) of rat tissue. N, normal. H, hyperthyroid A, avitaminous. Ordinate: mean $\pm 2 \times \text{S.E. of mean}$.

As with the pigeon, when the rat is showing avitaminous symptoms the cocarboxylase content of its tissues is much lower than normal. The values for the hyperthyroid animals (Fig. 3) are intermediate between those for the normal and the avitaminous ones.

Animals injected with vitamin B₁ daily

Table VII represents the values for animals which were given 250 $\mu\text{g.}$ vitamin B₁ daily by subcutaneous injection.

I. Animals fed on basal diet.

II. Animals fed on basal diet plus 0.4 g. desiccated thyroid per day.

Table VII. Cocarboxylase ($\mu\text{g./g.}$ fresh tissue) in tissues of rats injected daily with 250 $\mu\text{g.}$ vitamin B₁.

Exp. no.	Heart	Kidney	Liver	Brain
I. Normal animals				
79	7.51	3.82	6.38	2.53
80	9.01	4.24	9.81	2.80
87	7.10	3.91	7.24	2.21
II. Hyperthyroid animals				
41	7.82	—	6.71	—
75	8.01	3.02	4.41	—
76	8.63	3.10	3.70	—
82	7.76	3.25	6.21	1.89
83	9.04	3.88	4.01	2.51

The cocarboxylase values are much higher here than in those animals which were not injected with vitamin B₁. There was little difference between the values in normal and hyperthyroid animals except perhaps in the case of the liver. Thus it would seem that in the presence of excess vitamin B₁ thyroid feeding produces little diminution in the cocarboxylase content of the tissues.

Animals pretreated with vitamin B₁

Table VIII gives the average values for animals that had been pretreated with 250 µg. vitamin B₁ per day (subcutaneous injections) for 7 days.

I. Pretreatment for 7 days (basal diet plus 250 µg. vitamin B₁), then basal diet only.

II. Pretreatment for 7 days (basal diet plus 250 µg. vitamin B₁), then basal diet plus 0.4 g. desiccated thyroid per day.

Table VIII. *Average values for cocarboxylase content of tissues from rats pretreated with 250 µg. vitamin B₁ per day for 7 days (µg./g. fresh tissue).*

Group	Treatment	Tissue	No. of observations	Mean	2 × S.E. of mean
I	Pretreatment 7 days. Basal diet only	Heart	6	1.85	±0.18
		Kidney	6	0.90	±0.10
		Liver	6	1.35	±0.24
		Brain	6	1.92	±0.08
II	Pretreatment 7 days. Basal diet plus 0.4 g. desiccated thyroid per day	Heart	6	1.61	±0.20
		Kidney	6	0.71	±0.16
		Liver	6	0.95	±0.18
		Brain	6	1.47	±0.16

The individual results are given in the appendix (Tables XII and XIII).

The idea of the pretreatment was to increase the cocarboxylase of the tissues and then to trace the relative rate at which this level fell. It is seen that, especially in the case of liver and brain, the cocarboxylase values fell more rapidly in the hyperthyroid than in the normal animals. This difference is not so significant in the case of heart and kidney. Comparison with the figures of Table VII shows that even in the case of untreated animals the cocarboxylase values fall on a diet adequate but low in vitamin B₁. It is also seen that the figures of Table VIII are not greatly different from those of Table VI. The animals whose values are represented in Table VIII, however, were under treatment for a longer time.

DISCUSSION

The high levels of cocarboxylase found in various rat tissues after excess of vitamin B₁ had been provided in the diet confirms the results of Ochoa & Peters [1938]. A smaller supply of vitamin reduces the amount of cocarboxylase found, but not to a degree incompatible with normal health; this would be consistent with the view that the extra amounts were merely stores. We hesitate to use the word "saturation" which has acquired a special meaning, in connexion for example with vitamin C, but the analogy is close. It would seem that maximum possible levels of cocarboxylase are not necessary for full health.

Hyperthyroidism caused a fall in tissue cocarboxylase which was proved not to be due to a failure in phosphorylating capacity. The finding of low levels of brain cocarboxylase associated with catatorulin effects is consistent with previous work. It would be interesting to speculate upon the reasons for the diminution in tissue vitamin (combined or free), but in the absence of information as to why vitamin B₁ disappears in the course of metabolism, such speculations seem to be unprofitable; that the disappearance is due to excess metabolism in a wide sense seems likely. Such an explanation would be consistent with the reported changes in the concentrations of other vitamins in the tissues. We may instance the changes in the vitamin A stores (liver) following thyroid administration described by Abelin [1933] and Mlinko [1938], and in the vitamin C

stores (liver, adrenal cortex etc.) by Svirbely [1935], Nespor [1936], Mosonyi [1936], Thaddea [1938] and Mlinko [1938] using guinea pigs, and by Sure & Theis [1938; 1939] using rats. Protection from loss of weight in experimental hyperthyroidism with vitamin A has been described by Euler & Klussman [1932] and Logaras & Drummond [1938], and with vitamin C by Svirbely [1935], Demole & Ippen [1935] and Scafer [1936]. Drill & Sherwood [1938] also claim that other substances in yeast extracts lessen the fall in weight produced after maximal vitamin B₁.

The important point to realize is that the giving of thyroid or thyroxine to rats changes their normal requirements for vitamin B₁ in the sense that they need larger amounts, in proportion to the food eaten, to maintain proper tissue levels of vitamin. In the past [Kinnersley *et al.* 1928; Peters, 1930]¹ experimental evidence has been discussed for the existence of idiosyncrasies in the needs of similar animals for vitamin B factors. The suggestion arising now from the work of others (as well as ourselves) upon vitamin B₁ that such variations have an endocrine basis rests upon secure experiment for the first time. Further exploration is wanted, because a recognition of such individual variation in human requirements for various food elements might prove very significant.

SUMMARY

1. In confirmation of the work of others subcutaneous injection of vitamin B₁ gives protection from the loss in weight produced in rats by thyroxine injections. A pretreatment with vitamin B₁ also lessens the rate at which thyroid fed rats fall in weight.

2. Catatorulin effects are obtained with brain tissue from hyperthyroid animals but not from normal animals on the same diet.

3. Catatorulin effects, abolished by previous injection of the animal with vitamin B₁, are obtained with kidney tissue from both hyperthyroid and normal animals fed on a basal diet containing small but adequate amounts of vitamin B₁.

4. The cocarboxylase content of boiled tissue extracts from hyperthyroid animals is intermediate in value between those from normal animals and those from animals showing symptoms of vitamin B₁ deficiency.

5. Injection of vitamin B₁ increases the cocarboxylase content of the tissues of both normal and hyperthyroid animals.

6. After 7 days of pretreatment with vitamin B₁ the cocarboxylase content of the tissues falls more rapidly in hyperthyroid than in normal animals.

We are indebted to the Rockefeller Foundation for grants in aid of this work. Our thanks are due to Mr R. W. Wakelin for valuable technical assistance and to Miss Kempson for help with the rats.

¹ *Inter alia* they found that individual pigeons placed upon polished rice diet tended to develop symptoms in a remarkably constant time; they called this the "day constant".

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APPENDIX

Table IX. *Coccarboxylase* ($\mu\text{g./g. fresh tissue}$) in tissues of animals fed on basal diet

Exp. no.	Heart	Kidney	Liver	Brain
68	1.80	0.81	0.90	—
69	2.16	1.17	1.26	—
72	2.16	0.95	1.08	—
73	2.06	0.99	1.12	1.85
85	1.49	0.63	—	1.35
86	1.49	0.68	1.22	1.53
92	2.03	1.08	2.07	2.21
95	2.39	1.12	1.62	2.43

Table X. *Coccarboxylase* ($\mu\text{g./g. fresh tissue}$) in tissues of animals fed on basal diet plus 0.4 g. desiccated thyroid per day

Exp. no.	Heart	Kidney	Liver	Brain
56	1.08	0.45	0.58	—
57	1.57	0.95	0.77	—
61	1.12	0.50	0.23	—
84	1.08	0.50	0.41	0.81
88	1.13	0.41	0.59	1.22
89	1.26	0.50	0.54	1.40
93	1.21	0.89	1.08	1.62
94	2.03	0.81	0.99	1.89

Table XI. *Coccarboxylase* ($\mu\text{g./g. fresh tissue}$) in tissues of animals fed on avitaminous diet (animals showing symptoms)

Exp. no.	Heart	Kidney	Liver	Brain
77	0.36	0.23	0.41	0.81
77a	0.52	—	0.53	0.68
77b	0.31	—	0.36	0.53
92	0.22	0.36	0.31	0.77
93	0.45	0.40	0.49	0.89
94	0.61	0.23	0.50	1.04

Table XII. *Cocarboxylase ($\mu\text{g./g. fresh tissue}$) in tissues of animals pretreated for 7 days (basal diet plus 250 $\mu\text{g. vitamin B}_1$) followed by basal diet only*

Exp. no.	Heart	Kidney	Liver	Brain
90	2.12	1.04	1.66	1.84
91	1.80	1.04	1.40	2.11
91a	1.71	0.95	1.49	1.98
100	2.07	0.86	1.40	1.84
101	1.57	0.81	1.37	1.84
102	1.85	0.70	0.80	1.90

Table XIII. *Cocarboxylase ($\mu\text{g./g. fresh tissue}$) in tissues of animals pretreated for 7 days (basal diet plus 250 $\mu\text{g. vitamin B}_1$) followed by basal diet plus 0.4 g. desiccated thyroid per day*

Exp. no.	Heart	Kidney	Liver	Brain
90	1.30	0.54	0.88	1.57
91	1.53	0.53	1.04	1.56
91a	1.93	1.04	1.22	1.71
100	1.57	0.76	0.67	1.44
101	1.49	0.63	0.76	1.21
102	1.85	0.75	1.10	1.30

CXL. SOME FACTORS INFLUENCING THE FORMATION OF ROBISON ESTER FROM GLYCOGEN AND INORGANIC PHOSPHATE IN MUSCLE EXTRACT

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PARNAS & BARANOWSKI [1935], Ostern & Guthke [1936], Ostern *et al.* [1936] and Parnas & Ostern [1936], have shown that inorganic phosphate can react with glycogen in presence of muscle extract to form Robison ester, i.e. a mixture of reducing hexosemonophosphates, discovered in yeast by Robison [1922] and first found in muscle, where the % of the individual components differs from that in yeast, by Embden & Zimmermann [1927]. The hexosemonophosphate formation from glycogen was claimed to be direct and not a secondary phosphorylation of glucose formed by hydrolysing enzymes. Balance experiments showed that the final glycogen plus hexosemonophosphate could account for all the initial carbohydrate. The authors called the formation of Robison ester a phosphorylation of carbohydrate to distinguish it from the hydrolysis by amylase. Cori & Cori [1936; 1937], using washed muscle residue and well-dialysed extract, detected the formation of a hexosemonophosphate other than Robison ester from glycogen and inorganic phosphate. The new ester was non-reducing yet Robison ester was readily formed from it after addition of Mg. It was identified as glucose-1-phosphate [Cori *et al.* 1937]. The formation of the Cori ester is increased by adenylic acid: an action not yet quite explained, but apparently not due to phosphate transport by the adenylic acid. (The Coris call the enzyme producing the 1-ester phosphorylase; the one which produces the 6-ester from the Cori ester phosphoglucomutase.)

To study the formation of Robison ester in muscle extracts is of interest as this step may be a key reaction of carbohydrate breakdown, both oxidative and anaerobic [Engelhardt & Barchash, 1938]. In the latter case Robison ester is phosphorylated to hexosediphosphate and broken down to lactic acid: in the first it may be a substrate of Warburg's coenzyme the triphosphodihydropyridine nucleotide and eventually be linked up with atmospheric O₂ to be burnt to phosphohexonic-acid, phosphoketohexonic acid and arabinose phosphate + CO₂ [Warburg *et al.* 1935; Lipmann, 1936; see also Dickens, 1938, 2].

The investigation of conditions for hexosemonophosphate formation can be arbitrarily divided into two parts: (a) the kinetics of the reaction, and (b) the influence of activators and inhibitors.

(a) There can be no doubt that the step glycogen → Robison ester is not a true reversible equilibrium, as Robison ester added to muscle extract does not yield glycogen and inorganic phosphate under the same conditions as it is formed from the two compounds. Nevertheless, it was possible to show that in

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dialysed yeast juice as well as in dialysed muscle extract only a certain part of the glycogen was phosphorylated giving a relation of about 1 : 20 of (phosphorylated carbohydrate) to (unphosphorylated carbohydrate times free phosphorus) [Lehmann, 1938, 1]. The relation prevailed regardless of how much glycogen or phosphate was added. Furthermore, the same weight of starch was only half as much phosphorylated as glycogen, and it is known to have about double the number of monosaccharide units per chemically determined chain [see Bell, 1937]. The question is still controversial, for instance, glycogens of different chain length show no corresponding difference in their phosphorylation [Lehmann, 1938, 2].

Also in the presence of sufficient Mg, extracts transform all the glycogen into Robison ester provided that enough phosphate is present. These facts and the observation that glucose and hexosemonophosphate inhibit phosphorylation of glycogen [Lehmann, 1938, 1] led Cori *et al.* [1939, 1] to suggest that inhibition of phosphorylation by glucose-1-phosphate may play a part in cases where incomplete phosphorylation is observed. They find the Cori ester a specially strong inhibitor as compared with Robison ester. Kiessling [1939] (see also Schöffner & Specht [1938], Schöffner [1939]) was able to show the existence of a reversible equilibrium, glycogen + phosphate \rightleftharpoons Cori ester, by using purified yeast enzyme. This equilibrium can occur in muscle extract as well [Cori *et al.* 1939, 2] and may, under adverse conditions for the step Cori ester \rightarrow Robison ester, determine the end-point of the reaction glycogen + phosphate \rightarrow Robison ester.

(b) Several factors are known to influence lactic acid formation from carbohydrate in general. Phloridzin has been found [Lundsgaard, 1933] to act on the initial reaction, i.e. on phosphorylation. Others act on intermediate stages, while some have not yet been investigated as to the exact point where the inhibition or activation occurs.

It was felt that new knowledge of factors increasing or decreasing phosphorylation might be of interest for our understanding of carbohydrate breakdown in general, and might throw light on the conditions underlying the behaviour of weakly phosphorylating systems in particular. The following work was undertaken as an investigation of these influences in rabbit muscle extract; a subsequent publication will deal with the relation of these factors to phosphorylation in enzyme extracts from other species than rabbit and organs other than muscle.

METHODS

The tissue extracts used were all prepared according to Meyerhof [1926] by mixing two parts minced tissue with three parts water or 0.13% NaHCO_3 ; the suspension was filtered through muslin and inactivated for 1 hr. at room temperature. Most of the extracts were dialysed against water and KCl solutions at $+4^\circ$ for 48–60 hr. The degree of dialysis was tested by estimating the remaining inorganic phosphorus which was 0.5–5 mg./100 ml.

The glycogen used (British Drug Houses, Ltd.) was alkali-stable and did not contain reducing groups measurable under our conditions. The phosphate used was Sørensen buffer at pH 7. Mg was added as MgCl_2 , in concentrations of 0.05–0.1 mg. per ml. reaction mixture, while the adenylic acid used was the Na salt, in concentrations of 0.2–0.5 mg. per ml. of the reaction mixture. Except where otherwise stated the figures in this paper are calculated as mg. per ml. reaction mixture. The experiments were performed at 37° and were terminated by addition of trichloroacetic acid or strong KOH. The Robison ester formed was determined by measuring the loss of glycogen, the formation of new

difficultly hydrolysable phosphorus and reducing groups. The glycogen was estimated by Pflüger's method as modified by Good *et al.* [1933]. The phosphorus was determined after Fiske & Subbarow [1925] (see also Lohmann & Jendrassik [1926]) and hydrolysis of phosphorus esters was performed by Lohmann's principle [1928]. The reducing groups were measured according to Giragossintz *et al.* [1936] and Miller & Van Slyke [1936]. When this method is used the ratio of the reducing group of Robison ester measured as mg. glucose to the mg. P of the ester is 5.5 : 1. The Robison ester was isolated and identified after Ostern *et al.* [1936] on a large scale. The isolation on a micro scale after Cori & Cori [1931] was not performed in all experiments, but in all cases where a treatment led to an increase of Robison ester formation, the increase was confirmed by this method. The fraction isolated after Cori & Cori [1931] comprises both Cori and Robison esters. To obtain the true value for Robison ester it should be fractionated by hydrolysis. A necessary correction has to be made also for the P of adenylic acid present. Except where stated otherwise the amount of Cori ester present in our reaction-mixtures was negligible.

AMYLASES

Winfield & Hopkins [1915] observed that addition of pancreatic extracts to muscle "brei" caused an inhibition of lactic acid formation. Even a preparation of pancreatin, many years old, had the same inhibitory effect. Foster & Woodrow [1924] excluded both insulin and trypsin as the active principle. Ronzoni [1927] showed that lactic acid formation from glycogen was inhibited but not that from hexosephosphates. By using a cell-free enzyme solution from muscle prepared according to Meyerhof [1926] she found that the pancreatic extract acted by preventing the uptake of phosphate for esterification of glycogen. McCullagh [1928] confirmed her results and Case & McCullagh [1928] showed that the pancreatic factor was probably amylase. An inhibitory factor was found to be present in most tissues of the animal body, the strength of the inhibition corresponding to the amylase content and even plant amylases acted like pancreatic extracts.

The following experiments were performed to investigate whether this inhibition was due to the amylase itself or to its action on glycogen. In the latter case it had to be decided if the "amylase effect" was due either to a competition of phosphorylase and amylase for the substrate, or to the formation of an inhibitory compound formed from glycogen (it has been found, for instance, that glucose inhibited phosphorylysis [Lehmann, 1938, 1]).

Table I demonstrates that in the presence of different amylases the formation of Robison ester in muscle extracts was suppressed. The inhibition was less the more dilute the amylase solutions were.

In the experiments where amylase was present the glycogen did not remain unaffected, which showed that the amylase¹ was acting although phosphorylation was inhibited.

Trial experiments showed that a pH of 9 inhibited the rabbit liver amylase nearly completely but did not do the same to the phosphorylating enzymes of muscle extract. An experiment (Table II) at that pH was performed on the undialysed extract: the duration was 60 min., the initial glycogen 5.2 mg., and the reaction (pH 9) was obtained by $M/10 \text{ Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer.

¹ The amylase content of rabbit and hen liver varied greatly and in an unpredictable manner. The findings in guinea-pig liver were more constant.

Table I

All the enzyme solutions were undialysed, except in the first experiment, where the muscle extract used was dialysed 60 hr. The saliva used was always fresh. The duration of experiments was 60 min. except in no. 2, where it was 120 min. and in no. 3, where it was 20 min. In this, and all experiments unless otherwise stated, the figures indicate mg. per ml. of reaction mixture.

No.	Enzyme solution	Age of enzyme in days	Enzyme solution : total volume	Initial glycogen	Initial P	Robison ester formed
1	Rabbit muscle extract	18	1 : 6	10.7	1.1	3.1
	Rabbit liver extract	0.5	1 : 6	10.7	1.1	0
	Rabbit muscle extract	—	1 : 6	10.7	1.1	1.1
	+ rabbit liver extract	—	1 : 6			
2	Rabbit muscle extract	22	1 : 4	4	1.0	1.7
	Hon liver extract	1	1 : 3	4	1.0	0
	Rabbit muscle extract	—	1 : 4	4	1.0	0.7
	+ hon liver extract	—	1 : 3			
3	Rabbit muscle extract	2	1 : 3	8	1.2	1.9
	Human saliva	—	1 : 7	8	1.1	0
	Rabbit muscle extract	—	1 : 3	8	—	0
	+ saliva	—	1 : 7			
4	Rabbit muscle extract	3	1 : 3	8	1.2	4.7
	Human saliva	—	1 : 7	8	1.2	0
	Rabbit muscle extract	—	1 : 3	8	—	1.2
	+ human saliva	—	1 : 7			
5	Rabbit muscle extract	8	1 : 3	8	1.2	4.8
	Human saliva	—	1 : 7	8	1.2	0
	Rabbit muscle extract	—	1 : 3	8	—	4.3
	+ human saliva	—	1 : 600			
	Rabbit muscle extract	—	1 : 3	8	—	3.5
	+ human saliva	—	1 : 300			
	Rabbit muscle extract	—	1 : 3	8	—	2.7
	+ human saliva	—	1 : 150			

Table II

Enzyme solution	Age of enzyme in days	Enzyme solution : total volume	Initial P	Robison ester formed
Rabbit muscle extract	48	1 : 6	0.8	1.3
Rabbit liver extract	4	1 : 6	0.7	0
Rabbit muscle extract	—	1 : 6	0.9	1.8
+ rabbit liver extract	—	1 : 6		

Table II shows that when the liver amylase was inhibited at pH 9 there was no inhibition of phosphorylation. It is not known to what the increase in the presence of liver extracts was due. It is also demonstrated that the amylase inhibition of phosphorylation in the previous experiments was due to the action of the hydrolysing enzyme on glycogen, rather than to the presence of the amylase itself. The next experiments were done to see whether amylase formed an inhibitory product from carbohydrate or if it used up the glycogen so quickly that no substrate for phosphorylation was left. Even amylatic breakdown products from glycogen of high molecular weight are not phosphorylated by muscle extracts [Euler & Bauer, 1938]. In the case of an inhibitory compound being formed, therefore, in a reaction mixture, in which amylase and phosphorylase had been incubated together with glycogen, and in which the formation of Robison ester had come to an end, no new Robison ester should be formed after addition of more glycogen. In the case of competition of the enzymes for the substrate the same amount of Robison ester should be formed from the second addition of glycogen as was formed from the first.

Table III

The extracts used were undialysed; fresh human saliva was used.

Enzyme solution	Age in days	Enzyme solution : total volume	Duration of exp. in min.	Initial glycogen	Initial P	Remarks	Robison ester formed
Rabbit muscle extract no. 1	12	1 : 3	60	8	1.2	—	3.8
Rabbit muscle extract + saliva	12	1 : 3 1 : 750	60	8	1.3	—	1.9
Rabbit muscle extract + saliva	12	1 : 3 1 : 750	60	8	1.2	+ 8 mg. glycogen after 30 min.	3.0
Rabbit muscle extract no. 2	29	1 : 3.6	45	8.9	1.4	—	3.6
Rabbit muscle extract	29	1 : 3.6	140	8.9	1.4	—	4.7
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	45	8.9	1.4	—	2.6
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	140	8.9	1.4	—	3.4
Rabbit muscle extract + saliva	29	1 : 3.6 1 : 180	140	8.9	1.4	+ 8.9 mg. glycogen after 45 min.	4.7

The addition of more glycogen after the inhibition had manifested itself (Table III) was followed by a rise in phosphorylation. This could not have occurred if the previous suppression of that process had been due to formation of an inhibitor, e.g. glucose. The amylatic inhibition of phosphorylation may therefore be conceived as due to lack of substrate for the phosphorylase due to the removal of the substrate by the hydrolysing enzyme.

OXIDATION

Another inhibitory factor for phosphorylating anaerobic breakdown of carbohydrate is the presence of O_2 or oxidation. Pasteur found that the fermentation of yeast was suppressed by O_2 and a similar inhibition of lactic acid formation in intact muscle has been described by Meyerhof. That muscle extract is able to glycolyse vigorously in the presence of air is due to the fact that the extract lacks the O_2 carriers and activators which are present in intact muscle tissue. Lipmann [1933] was able to show a reversible inhibition of glycolysis in extracts by dyes which linked up the oxidizable groups of the muscle extract with atmospheric O_2 . Lately, Rapkine [1938] has demonstrated that the oxidation of triosephosphate and hence the oxidoreduction between triosephosphate and pyruvic acid which occurs midway in the glycogen \rightarrow lactic acid reaction, is inhibited by oxidizing agents such as oxidized glutathione and I_2 . Previously Lipmann [1934] had described an inhibition of glycolysis by $CuSO_4$ and Gemmill & Hellerman [1937] by Hg^{++} salts. The latter obtained indications that the inhibition occurred probably at the "hexose stage". The inhibitions could be removed by reducing agents such as reduced glutathione and cysteine [Wagner-Jauregg & Rzeppa, 1936, 1, 2; Gemmill & Hellerman, 1937].

The following data are given to show the inhibiting effect of oxidizing compounds on Robison ester formation from glycogen and inorganic phosphate. As examples are given a biological oxidizing system (catechol + catechol oxidase) and oxidized glutathione. The catechol oxidase was prepared from mushrooms following advice from Dr K. Baghvat. We also obtained a highly purified sample from Dr T. Mann prepared by the method of Keilin & Mann [1938].

Table IV

25 days old, 72 hr. dialysed rabbit muscle extract. 1 : 4 of total volume.
45 min. duration of exp. Initial glycogen 6 mg. Initial P 0.75 mg.

Additions	Robison ester formed
—	2.5
+ Catechol oxidase	2.5
+ <i>M</i> /20 catechol	1.3
+ Catechol oxidase + <i>M</i> /20 catechol	0

20 days old undialysed rabbit muscle extract. 1 : 3 of total volume.
30 min. duration of exp. Initial glycogen 8 mg. Initial P 1.2 mg.

—	2.8
<i>M</i> /80 GSSG	2.3

2 days old, 60 hr. dialysed rabbit muscle extract. 1 : 3 of total volume.
120 min. duration of exp. Initial glycogen 6.25 mg. Initial P 0.4 mg.

—	1.65
<i>M</i> /24 GSSG	1.0
<i>M</i> /12 GSH	2.2

In the last experiment of Table IV the function of GSSG as inhibitor was controlled with a reduced sample of the same preparation. This was to exclude the action of any traces of Cu which might have been present in small amounts owing to its method of preparation. The reduced tripeptide not only had no inhibitory effect but actually increased the formation of Robison ester.

ADRENALINE

Instead of catechol, in the experiments when catechol oxidase was used, adrenaline may be added together with the oxidase to give an inhibitory effect. Adrenaline is even more suitable as it has no inhibitory effect by itself, unlike catechol which is never entirely free from oxidized forms and undergoes also a slight autoxidation under the conditions of our experiment. The only effect observed of adrenaline on Robison ester formation was this inhibitory one; high concentrations were necessary (0.2–1 %) and an oxidizing enzyme. At alkaline reaction the autoxidation of high concentrations of the hormone was sometimes sufficiently strong to effect an inhibition by itself. Smaller amounts of the hormone nearer physiological concentrations were never found by us to have an influence on the enzyme system forming Robison ester from glycogen. Our findings are in agreement with the results of Cori *et al.* [1939] on extracts.

REDUCING AGENTS

In Table IV an experiment was shown where reduced glutathione increased Robison ester formation. We were able to confirm that GSH in particular and reducing agents in general have an effect on Robison ester formation the reverse of that of oxidizing agents.

The estimation of reducing groups of the ester was, of course, not possible in the presence of other reducing compounds and had to be performed after isolation [Cori & Cori, 1931]. This was done in the presence of all individual reducing agents tested, but in most of the experiments reported in this section the glycogen loss and the formation of difficultly hydrolysable P were taken as a basis for the measurement of Robison ester.

Reduced glutathione

Table V

In both experiments 60 hr. dialysed, 9 and 10 days old rabbit muscle extract was used. Extract : total volume = 1 : 4. Initial glycogen 6 mg. Initial P 0.8 mg. pH 8.5 was obtained by the use of Na_2HPO_4 as a source of phosphate and was maintained by NaHCO_3 buffer.

Duration of exp. min.	pH	Addition	Robison ester formed
60	7	—	2.4
60	7	M/25 GSH	3.3
60	8.5	—	3.4
60	8.5	M/25 GSH	4.2
75	8.5	—	3.1
75	8.5	M/10 GSH	4.1

In all cases reduced glutathione increased Robison ester formation and it will be noted that whereas an alkaline reaction itself seems to have a favourable effect, the action of glutathione is evident at either neutral or alkaline reaction. Dyes inhibiting fermentation of yeast and oxidizing agents suppressing glycolysis of muscle extract might do so by oxidizing the enzyme necessary for the Robison ester formation, and reduced glutathione might be considered as an activator, reducing the inactive enzyme. It is known already that GSH can reduce proteins [Hopkins & Dixon, 1922; Hopkins, 1925] and enzymes (dehydrogenases) of carbohydrate metabolism [Rapkine, 1938; Hopkins & Morgan, 1938; Hopkins *et al.* 1938].

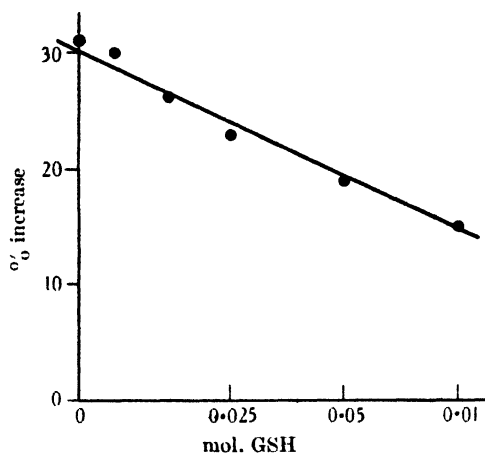


Fig. 1. % increase of Robison ester formation at different concentrations of reduced glutathione.

Fig. 1 shows that the % increase of Robison ester due to glutathione is in a logarithmic relationship to the concentration of the tripeptide. The graph is based on the last experiment in Table V. The Robison ester formation in absence of any glutathione was 3.1 mg. (= 100 %).

Cyanide

The action of reduced glutathione may be visualized as a reduction of SS groups of the muscle extract. The influence of KCN on phosphorylation was therefore investigated, since according to Mauthner [1907] it is a reducing agent in the sense that it transforms SS linkages into free SH groups.

Table VI

In all cases rabbit muscle extract was used with adenylic acid and Mg unless otherwise stated.

20 days old undialysed extract. Enzyme solution : total volume = 1 : 3.

Duration of exp. 20 min. Initial glycogen 8 mg. Initial P 1.1 mg.

Remarks	Robison ester formed
—	2.9
M/10 KCN	5.2

9 days old, 48 hr. dialysed extract. Enzyme solution : total volume = 1 : 3.

Duration of exp. 100 min. Initial glycogen 4 mg. Initial P 0.5 mg.

—	3.2
M/10 KCN	3.6

Fresh undialysed extract. Enzyme solution : total volume = 1 : 4.

Duration of exp. 60 min. Initial glycogen 6 mg. Initial P 0.9 mg.

—	4.9
M/7 KCN	5.7

15 days old, 60 hr. dialysed extract. Enzyme solution : total volume = 1 : 4.

Duration of exp. 180 min. Initial glycogen 6 mg. Initial P 0.8 mg.

No adenylic acid and Mg added 0.7

No adenylic acid and Mg added, 1.7

M/2 KCN

—	4.0
M/2 KCN	5.7

It will be noted that KCN increases Robison ester formation in old as well as fresh extracts. Its effect is evident both with and without addition of adenylic acid and Mg^{++} . The influence of K^+ of the KCN was checked with equivalent amounts of KCl. Mg^{++} may, in its effect on formation of Robison ester, be replaced by other positive ions [Cori *et al.* 1938] and Ohlmeyer & Ochoa [1937] working on yeast enzymes included K^+ in the list. The chloride was chosen because the KCN used by us was neutralized with HCl. KCl had no effect on the formation of Robison ester.

Thiolacetic acid

Thiolacetic acid added as the Na salt behaved similarly to the other reducing compounds investigated.

Table VII

28 days old undialysed rabbit muscle extract. 1 : 4 to total volume.

6 mg. initial glycogen, 0.8 mg. initial P.

Time of exp. min.	Robison ester formed	
	Control	N/10 thiolacetate
10	1.0	2.2
15	2.4	4.7
100	3.5	5.3

Denatured protein (boiled muscle extract)

Boiled protein may contain SH groups formed during the process of denaturation [Harris, 1923; Hopkins, 1925]. There are exceptions, but muscle extract is not one, as can be demonstrated by means of the nitroprusside test.

The increasing effect on lactic acid formation and fermentation of boiled muscle extract was recognized quite early as not necessarily due to protein [Meyerhof, 1918, 1, 2; Meyer, 1927] but to inorganic phosphate [Meyer, 1927; 1928], Mg and adenylypyrophosphate [Lohmann, 1931] and reduced codehydrogenase I [Harden-Euler's coenzyme] [Meyerhof & Ohlmeyer, 1936]. On the other hand, Kendal & Stickland [1937] observed an activation of diluted muscle extract with boiled dialysed muscle extract which was not shown by the dialysable compounds only. The point was made by Curtius & Ohlmeyer [1938] that some enzymes of muscle extract are remarkably resistant towards boiling and Kendal & Stickland [1937] may therefore have merely added active enzyme to the diluted extract. We are able to confirm both views. The Heidelberg investigators only observed the insensitiveness of the Parnas reaction enzyme of muscle to boiling; we found the same regarding the enzymes responsible for the formation of Robison ester from glycogen and inorganic phosphate.

Muscle extract boiled for 2 min. is capable of phosphorylating glycogen and forming Robison ester and the findings of Kendal & Stickland [1937] may thus find their explanation. The resistance of muscle extract to boiling is a phenomenon which we could not always repeat but the exceptions were few.

Table VIII. *Influence of muscle extract kept for 2 min. at 97°*

Total extract : total volume	Unboiled extract : total volume	Boiled extract : total volume	Robison ester formed
24 days old, 60 hr. dialysed rabbit muscle extract, boiled and unboiled.			
Duration of exp. 135 min. Initial glycogen 4 mg. Initial P 0.9 mg.			
1 : 4	1 : 4	—	1.4
1 : 4	—	1 : 4	0.7
1 : 2	1 : 4	1 : 4	2.3
27 days old, 100 hr. dialysed rabbit muscle extract, boiled and unboiled.			
Duration of exp. 100 min. Initial glycogen 6 mg. Initial P 0.7 mg.			
1 : 2	1 : 2	—	2.5
1 : 4	1 : 4	—	1.8
1 : 2	—	1 : 2	1.3
1 : 2	1 : 4	1 : 4	2.5

Table IX. *Influence of globulin x on phosphorylation in rabbit muscle extract*

Extracts to total volume 1 : 4; initial glycogen 6 mg.	
7 days old, 60 hr. dialysed extract. Duration of exp. 90 min. Initial P 0.8 mg.	
Additions	Robison ester formed in mg.
3% globulin x	4.0
3% denatured globulin x	4.4
8 days old, 60 hr. dialysed extract. Duration of exp. 90 min. Initial P 0.8 mg.	
—	3.3
3% globulin x	3.8
3% denatured globulin x	4.3
8 days old undialysed extract. Duration of exp. 90 min. Initial P 0.9 mg.	
3% globulin x	6.8
3% denatured globulin x	7.5
18 days old, 60 hr. dialysed extract. Duration of exp. 210 min. Initial P 0.7 mg.	
—	3.6
2% globulin x	3.6
2% denatured globulin x	4.4

Yet we were able to confirm Kendal & Stickland's view [1937] that boiled muscle extract may contain an activator not present in diluted muscle extract. This activator may actually be formed during boiling. A single muscle protein fraction "globulin x" though unable to phosphorylate glycogen, may sometimes slightly increase phosphorylation of muscle extracts and this influence is greatly increased after it has been denatured by boiling. At the same time a formation of SH groups after boiling was shown to have occurred by means of the nitroprusside test. The fact that the "globulin x" acts sometimes as an activator, even before being boiled, may well be due to some denaturation which has taken place already, as the purified protein denatures on standing. We are greatly obliged to Dr E. C. Smith of the Low Temperature Station, Cambridge, for repeated gifts of globulin x.

Sodium hydrosulphite

An inorganic reducing agent $\text{Na}_2\text{S}_2\text{O}_4$ was also shown to increase phosphorylation (Table X).

Table X

27 days old undialysed rabbit muscle extract, 1 : 3 total volume. Duration of exp. 65 min.
Initial glycogen 8 mg. Initial P 1.2 mg.

Additions	Robison ester formed
—	1.8
M/45 $\text{Na}_2\text{S}_2\text{O}_4$	2.6
M/135 $\text{Na}_2\text{S}_2\text{O}_4$	2.4

Evacuation

Physical deprivation of O_2 was chosen as another example of reduction of muscle extracts. To avoid any difference of the buffering of the extract by alteration of its CO_2 content all samples in the experiments were first thoroughly evacuated in Thunberg tubes. But whereas some of them were left so during the experiment, others were reopened and shaken up with air.

Table XI

Hen breast muscle extract, 11 days old, undialysed. 8 mg. initial glycogen,
0.8 mg. initial P.

Duration of exp. min.	Special conditions	Robison ester formed
60	In air	2.2
60	<i>In vacuo</i>	2.4
120	In air	3.1
120	<i>In vacuo</i>	3.7

Table XI shows that there is a very slight increase of Robison ester formation *in vacuo*.

METALS

The effect of SH groups on enzyme function has often been studied simultaneously with that of metallic ions. Work mentioned above [Lipmann, 1934; Gemmill & Hellerman, 1937 etc.] was done in connexion with the inhibition of fermentation and lactic acid formation by oxidizing agents showing that instead of dyes, I_2 , GSSG or quinone, the higher valent forms of metals which exist in different valency states may be used. It was to be expected, therefore, that Cu^{++} for instance, would inhibit Robison ester formation just as oxidized glutathione

does. Many authors, especially Hellerman & Perkins [1934; 1935], have shown that several enzymes involved in protein metabolism may be influenced by metals not only by oxidation of their SH groups or reduction of SS groups, but also by formation of metallic derivatives. We therefore investigated the effect of various metallic ions on the formation of Robison ester.

Again, it was not possible to estimate the reducing groups of the Robison ester even after the micro isolation of Cori & Cori [1931], as sufficient metal to upset the estimation remained in the isolated fraction. But the glycogen loss and formation of difficultly hydrolysable phosphate were in agreement and the organic phosphate formed was refound in the hexosemonophosphate fraction by the method of Cori & Cori [1931].

Table XII. *Influence of metals on Robison ester formation*

60 hr. dialysed rabbit muscle extract. Extract : total volume = 1 : 4. Initial glycogen 6 mg., initial P 0.8 mg. 0.5 mg. adenylic acid. 0.05 mg. Mg. Na_2SO_4 was used for control values.

Age of extract in days	13	16	13	17	18	19	23	23
Duration of exp. in hr.	2	2	2	2	2	2	5	5
Molarity of metal or Na_2SO_4	1×10^{-2}	5×10^{-3}	1×10^{-3}	5×10^{-4}	5×10^{-5}	1×10^{-5}	1×10^{-6}	1×10^{-7}
mg. Robison ester formed in presence of Na_2SO_4	2.1	2.1	2.1	2.5	2.2	2.1	4.4	4.4
\pm % in presence of CuSO_4	-60	-78	-60	-95	-66	-34		
\pm % in presence of $\text{Fe}_2(\text{SO}_4)_3$	-	-73	-	-15	-9	± 0		
\pm % in presence of FeSO_4	-57	-29	-12	-13	-			
\pm % in presence of ZnSO_4	-100	-81	-26	-30	-32	-14	0	+7
\pm % in presence of NiSO_4	+40	+40	+51	+19	+6	-		
\pm % in presence of $\text{Co}(\text{NO}_3)_2$	+20	+38	+51	+32	+26	+24	+10	± 0

In the experiments shown in Table XII no increase of phosphorylation was obtained with the ferrous salt, though the inhibition here was slightly less than in the case of the ferric salt. In low concentrations the ferrous salt is of course unstable.

As the cobalt salt used was $\text{Co}(\text{NO}_3)_2$ controls were performed with NaNO_3 which, however, had no influence on Robison ester formation. (It will be noted that an optimal concentration of Mg^{++} is present and the influence of Co^{++} can therefore not be explained as a replacement of Mg^{++} . Co^{++} can take the place of Mg^{++} in well dialysed enzyme preparations [Cori *et al.* 1938; 1939, 1].)

The influence of a metal ion is generally the same in the range of concentrations given although Co^{++} may sometimes inhibit at 10^{-2} M and Zn^{++} may activate rather than inhibit at very low concentrations. But whereas a qualitative consistency of the influence of the various metals on different muscle extracts can be observed, there is a great inconsistency in the quantitative effect. 10^{-3} M Co^{++} may increase the Robison ester formation in one extract 200%, in another only 30%. Co^{++} and Zn^{++} in equal molecular concentrations counteract each other, as shown in Table XIII.

The action of Co^{++} was further elucidated (in experiments which will appear fully in a subsequent publication) as being more than a mere replacement of Mg^{++} , for Co^{++} increased the phosphorylating power of weakly phosphorylating

Table XIII

50 hr. dialysed, 21 days old rabbit muscle extract. 0.05 mg. Mg, 0.5 mg. adenylic acid. Initial glycogen 6 mg. Initial P 0.8 mg. Robison ester formed 1.6 mg. in 4 hr.

Molarity of metal	± % Robison ester formation in presence of metals				
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Zn	-25	-13	± 0	± 0	+6
Co	+ 5	+ 5	+ 5	+5	± 0
Co	± 0*	± 0*	-13*	—	—

* 10⁻⁴ M Zn present.

enzyme systems, although Mg⁺⁺ had no influence on them [see also Gill & Lehmann, 1939]. It may act either as a complex former in the sense of Hellerman & Perkins' [1935] suggestion of enzyme-metal complex formation, or as a reducing agent in the sense of Michaelis and his collaborators. They observed that Co⁺⁺ combines with cysteine with subsequent uptake of O₂ [Michaelis *et al.* 1929; Michaelis & Yamaguchi, 1929]. Rosenheim & Davidsohn [1904] showed formation of a complex of thiolacetic acid with Co⁺⁺ and with Zn⁺⁺. Yet we find a difference in the interactions between Zn⁺⁺ and Co⁺⁺ with thiolacetic acid.

When Zn⁺⁺ or Co⁺⁺ are added to thiolacetic acid at neutral reaction, a loss of power of the thiolacetic acid to consume I₂ can be demonstrated in the case of Co⁺⁺ but not in the case of Zn⁺⁺. This is shown in Table XIV.

Table XIV. *Effect of Co on I₂ consumption of thiolacetic acid*

Reaction mixture	Consumption of N/200 N I ₂ (ml.)
1 ml. M/20 thiolacetic acid	4.3
5 ml. M/10 phosphate (pH 7)	
3 ml. 25% KI	
Ditto + 0.5 ml. M/10 Co(NO ₃) ₂	1.5
Ditto + 0.2 ml. M CO(NO ₃) ₂	1.3
5 ml. M/10 phosphate (pH 7)	0.01
3 ml. 25% KI	
0.2 ml. M Co(NO ₃) ₂	

The most plausible explanation perhaps is a combination of the views of the Baltimore and New York workers in suggesting a specific and powerful reduction of the enzyme through complex formation with the metal.

ASCORBIC ACID

Ascorbic acid is a reducing compound and might be expected as such to increase phosphorylation. But, whereas we had one experiment when ascorbic acid acted in this way, we were never able to repeat it and we found rather that ascorbic acid in large amounts inhibited phosphorylation. Its inhibitory effect occurred in concentrations similar to those required for glucose to exert an inhibitory effect, but its inhibition was only one-quarter of that of glucose. It may join, therefore, the other similarly acting unphosphorylated carbohydrates such as mannose and dimeric glyceraldehyde [Lehmann & Needham, 1938].

Yet the influence of ascorbic acid is dependent on pH whereas that of glucose is not. Ascorbic acid is more inhibiting at pH 8 than at pH 7. A destruction of the compound at neutral pH could be excluded: we are obliged to Mr Crook

Table XV

7 days old, 60 hr. dialysed rabbit muscle extract. Extract:total volume = 1:4. Initial glycogen 6 mg. Initial P 0.8 mg. Duration of exp. 130 min. Robison ester formed 3.3 mg.

% Glucose	Inhibition in %	% Ascorbic acid	Inhibition in %
1	- 55	1	- 15
0.8	- 53	0.8	- 17
0.6	- 46	0.6	- 15
0.4	- 40	0.4	- 5
0.2	- 15	0.2	- 4
0.8% glucose + 0.2% ascorbic acid	- 54	0.8% ascorbic acid + 0.2% glucose	- 36

who measured ascorbic acid at the beginning and end of several of our experiments. The reason for a stronger inhibition at pH 8 may be the formation of oxidation products from ascorbic acid.

INSULIN

Insulin as a SS compound would be expected to act as an inhibitor. This it actually does [Lehmann, 1938, 2], but at a concentration too small for its effects to be comparable with that of SS glutathione. Cori *et al.* [1938] suggested that the Zn content of commercial preparations may have been the reason for Lehmann's [1938, 3] findings. They were able to repeat them with a commercial preparation but not with a specially purified sample. The insulin used was a British Drug Houses sample of the crystalline hormone which contained insufficient Zn to inhibit after the insulin was destroyed by reduction. It might be questioned also if the combined Zn of crystalline insulin can act at all like the free ion. Furthermore, insulin hydrochloride acts as an inhibitor as well and the following experiment shows a comparison of the effect of different samples. Insulin which contained only 0.018% Zn was very kindly provided by Messrs Boots Drug Co. Ltd. The final concentration in the experiment was therefore 0.07 $\mu\text{g./ml.}$ or 10^{-6} M.

Table XVI. *Different samples of insulin*

5 hr. dialysed rabbit muscle extract, fresh. Extract to total volume - 1:4. Initial glycogen 2.5 mg./ml. Initial P 1.2 mg. ml. 0.25 mg. adenylic acid. No extra Mg added.

Addition of 0.4 mg. Insulin $\cdot 1.3 \cdot 10^{-5}$ M	Robison ester formed	Inhibition
--	1.1	--
Crystalline insulin (Zn insulinate)	0.7	35%
Insulin hydrochloride (Zn-poor)	0.7	
Commercial insulin hydrochloride	0.7	

There is no difference in the inhibitory action of the different samples of insulin, whether they are Zn insulinate or not. The analysis showed that glycogen

Table XVII. *Accumulation of Cori ester in the presence of insulin*

4 days old undialysed rabbit muscle extract. No adenylic or Mg added. Initial glycogen 8 mg. Initial P 0.8 mg. Duration of exp. 20 min.

Addition	Total hexosemono- phosphate	Cori ester	Inhibition of Robison ester formation
--	3.3	0	--
0.07% crystalline insulin	1.9	0.4	50%

and Robison ester could not account for all the carbohydrate in our experiments and an accumulation of Cori ester was observed. Insulin inhibited the formation of Robison ester but allowed an accumulation of Cori ester.

The inhibition by insulin can be counteracted by increasing the Mg in the reaction mixture [Lehmann, 1938, 4]. On the other hand in well dialysed muscle extracts to which Mg is not added it inhibits little of the small phosphorylation going on, but after addition of Mg the increase due to the ion can be suppressed by insulin.

Table XVIII

48 hr. dialysed, 12 days old rabbit muscle extract. Extract: total volume = 1:4. 6.5 mg. adenylic acid added. Initial glycogen 6 mg. Initial P 1.2 mg. Duration of exp. 30 min. The insulin added was neutralized with NaOH.

mg. Mg added	mg. insulin hydrochloride added	Total hexosemono- phosphate formed	Robison ester formed
—	—	2.6	1.1
—	4.5	2.3	1.2
0.1	—	4.0	3.6
0.1	4.5	3.0	1.9

There seems, therefore, to be an interaction between insulin and Mg^{++} taking part in the insulin inhibition. The effect cannot be demonstrated either in the presence of too much or of too little Mg^{++} at a given concentration of insulin (0.3–1 %). This interaction can be observed in model experiments. At a neutral to slightly alkaline reaction Mg^{++} forms a precipitate with insulin. The precipitate cannot be observed in a 0.3 % insulin solution with Mg^{++} concentration below 0.01 mg./ml. If more is added an effect takes place which may be described as follows. The Mg^{++} becomes attached to the negatively charged protein molecule. If enough Mg^{++} is present the negative charges are neutralized and the protein molecule now forms an isoelectric precipitate. Much Mg^{++} leads to resolution due to the zwitterion effect.

Thus in tissue extracts a given amount of insulin at about neutral pH will precipitate Mg^{++} . No precipitation occurs if the concentration of Mg^{++} is diminished by dialysis or if a further addition of Mg^{++} has caused resolution of a previously formed precipitate. A similar effect of phosphate on the solubility of haemoglobin has been described and its theory has been explained by Adair & Adair [1934].

The insulin effect observed is not specific; instead of Mg^{++} any other divalent positive ion can be chosen and insulin may be replaced by a protein of a similar isoelectric point.

Insulin reduced by cyanide neither inhibits phosphorylation, nor does it at a neutral pH show the same effect on a Mg^{++} solution. This may be an explanation of the fact that the insulin inhibition wears off with the duration of the experiment [Lehmann, 1938, 2]. It is possible to demonstrate that insulin is reduced during incubation with muscle extract [Lehmann & Schlossmann, 1938], by separating the insulin from the muscle extract by short heating with subsequent centrifuging. The insulin stays in solution while the muscle proteins are precipitated. The reduction can be shown furthermore by using a washed suspension of minced muscle according to the technique of Hopkins & Dixon [1922] for showing reduction of glutathione by muscle proteins. In this method no boiling is needed before centrifuging. It may be mentioned that reduced insulin is precipitable with trichloroacetic acid and can therefore be separated

from cysteine and glutathione which may have been liberated from muscle proteins during incubation under the influence of insulin; and which compounds might show a positive SH test in the insulin fraction.

SUGARS

The inhibition of Robison ester formation from glycogen by glucose seems to be an indirect one. We found that glucose does not inhibit the formation of Robison ester from Cori ester. (We are grateful to Dr B. E. Holmes for providing us with a sample of the K salt of the Cori ester prepared after Kiessling [1938].) Table XX shows the effects of various sugars on phosphorylation. The inhibition with mannose is only demonstrable with a freshly dissolved sample, a few days standing in the ice chest causing the inhibitory power to disappear. The effect of glucose is in agreement with Haarmann & Stratmann's [1932] finding that 0.5% glucose inhibits the lactic acid formation from glycogen in skeletal muscle. They observed a great individual variety in the degrees of inhibition. We too have to report that the inhibition of phosphorylation varies at constant glucose concentrations. Most extracts are inhibited from 50 to 80% at 1% glucose concentration, but we had several extracts which were inhibited only 20% and higher concentrations of hexose did not increase the inhibition further.

Table XIX. *Phosphate uptake in presence of glycogen and various sugars*

All values expressed as mg. in total reaction mixture.

1 ml. 60 hr. dialysed, 21 days old rabbit muscle extract. 2 ml. additions containing: 0.5 *M*/15 phosphate, 20 mg. glycogen, 1 mg. adenylic acid (Na salt). 0.2 mg. Mg as $MgCl_2$. Initial P 1.15 mg. Duration of exp. 120 min. Total volume of reaction mixture: 3 ml.

Addition	Inorganic P found (mg.)	P uptake mg.
—	0.68	0.47
1% Fructose	0.71	0.44
1% Glucose	0.99	0.16
1% Mannose	0.95	0.20
1% Galactose	0.83	0.32
1% Lactose	0.76	0.39
1% Arabinose	0.71	0.44
2% Maltose	0.79	0.36
2% Sucrose	0.77	0.38
3% Raffinose	0.66	0.48

Table XX. *Phosphate uptake inhibited by Robison ester and glucose*

1 ml. 11 days old, 60 hr. dialysed rabbit muscle extract. 2 ml. additions containing 0.5 ml. *M*/15 phosphate (pH 7), 10 mg. glycogen, 1 mg. adenylic acid (Na salt). 0.3 mg. Mg as $MgCl_2$. Duration of exp. 120 min. Initial P 1.15 mg.

Additions	Inorganic P found (mg.)	P uptake mg.
—	0.37	0.78
0.6% Glucose	0.81	0.34
1.0% Robison ester*	0.75	0.40

* The Robison ester was free from inorganic P.

Hexosediphosphate differs from other sugars inasmuch as at $3-1 \times 10^{-3}$ *M* it stimulates the phosphorylation of glycogen. The increase in Robison ester formation is not inhibited by iodoacetate or arsenate. The latter especially would poison specifically all indirect phosphorylation due to oxido-reduction of triosephosphate [Needham & Pillai, 1937].

Table XXI. *Increase of Robison ester formation in the presence of hexosediphosphate*

2 days old undialysed rabbit muscle extract. Extract to total volume = 1 : 2. Initial glycogen mg. Initial P 1.9 mg. Duration of exp. 40 min.

Addition	Robison ester formed (mg.)	
	<i>M</i> /400 iodoacetate	<i>M</i> /100 sodium arsenate
—	2.8	2.2
<i>M</i> /200 Hexosediphosphate	7.2	5.6

Both iodoacetate and arsenate have at high concentrations a diminishing effect on Robison ester formation, which should be distinguished from a specific inhibition. The inhibition of Robison ester formation by iodoacetate (Table XXI) was 18% in absence, 20% in presence of hexosediphosphate; arsenate inhibited in both cases by 40%.

Kendal & Stickland [1938] claimed that it was necessary to have traces of hexosediphosphate present for the action of the enzyme converting Cori ester into Robison ester. This was not confirmed by Cori *et al.* [1938]. The effect observed by us is not identical with that of Kendal & Stickland. Hexosediphosphate seems to act on the initial phosphorylation of glycogen. Its action results first in an accumulation of Cori ester which subsequently is slowly transformed into Robison ester as is shown in Table XXII.

Table XXII

6 days old undialysed rabbit muscle extract. Extract : total volume = 1.5 : 2. Initial glycogen 6 mg. Initial P 0.8 mg.

Time of exp. min.	Total hexosemono-phosphate formed mg.	Robison ester formed mg.	Cori ester formed mg.	Addition
10	1.1	1.0	0.1	
	2.8	1.2	1.6	<i>M</i> /300 Hexosediphosphate
30	1.2	1.2	—	
	2.8	1.9	0.7	<i>M</i> /300 Hexosediphosphate
120	2.2	2.2	—	
	2.9	2.9	0	<i>M</i> /300 Hexosediphosphate

Negelein & Brömel [1939] observed that glyceraldehyde phosphate in presence of a purified yeast enzyme combines with inorganic phosphate to form a triosediphosphate. This can transfer one phosphate molecule to glucose. Hexosediphosphate added to muscle extract yields glyceraldehyde phosphate [Meyerhof & Lohmann, 1934] and a reaction in muscle extract may take place like the one described in yeast. Under the conditions of our experiments neither hexosediphosphate nor triosephosphate were themselves phosphorylated. Yet ordinary yeast juice does not show this process either and a purification of the enzymes concerned may be necessary for isolating this phosphorylation.

DISCUSSION

The fact that amylases compete with phosphorylating enzymes for the substrate glycogen supports the view that there are at least two possible paths of anaerobic glycogen breakdown (see especially Parnas [1937]). The question

arises as to whether these two systems can act in muscle side by side. Whereas Parnas [1937] denies the occurrence of amylases in muscle, they have been described repeatedly by other authors [see Lohmann, 1926; Willstätter & Rohdewald, 1938]. Lately, Augustin [1938] reported from Parnas' laboratory the existence of amylase and phosphorylase side by side in heart muscle of several mammals. The contradictory statements for skeletal muscle may find an explanation in the individual variation of the amylase content of skeletal muscle in the same species of animals, a fact which we observed specially in frog muscle.

In a subsequent publication we will deal with the biochemical background for the poor phosphorylating power of certain tissues [see also Gill & Lehmann, 1939]. We observed that some frog muscle extracts contain very little amylase, others very much. The phosphorylation in these extracts was in inverse proportion to their amylase activity. Case [1931] observed a loss of I_2 coloration after incubation of glycogen with boiled muscle tissue. No lactic acid was formed. He concluded that there were amylases acting on the glycogen. Our findings regarding the heat stability of the enzymes involved in the formation of Robison ester suggest that a formation of Robison ester might also have occurred. Yet we ourselves found a loss of I_2 staining power in glycogen after a short incubation with rabbit muscle extract followed by isolation using Pflüger's method. The loss of I_2 staining power did not correspond to the formation of hexosemonophosphate or the loss of glycogen, that is to say, the same amount of glycogen, isolated according to Pflüger, had less I_2 staining power after incubation with rabbit muscle extract than it had before. For starch, the I_2 coloration has been found by Hanes & Cattle [1938] to be a reliable index of amylatic degradation.

The experimental data of this paper seem to indicate that Robison ester may not be an intermediate of the oxidative breakdown of carbohydrate.

The fact that oxidation inhibits its formation and that reduction increases it has only been established for extracts. The possibility cannot be excluded that very fresh not inactivated extracts and especially the enzyme of the living cell never attain a state of oxidation sufficient to prohibit the Robison ester formation. Rapkine [1938] excluded the formation of phosphoglyceric acid from triosephosphate in oxidized muscle extract. We suggest now that Robison ester is one of the compounds probably not formed from glycogen and inorganic phosphate in oxidized muscle. Whether or not phosphorylation plays any part in oxidative carbohydrate breakdown cannot be decided before more is known of the enzymic oxidation of the Cori ester. The phosphorylation of glycogen by inorganic phosphate through adenylypyrophosphate is a possibility which should not be excluded [Lehmann & Needham, 1937]. Yet it is improbable that it occurs in the process of oxidation, as the source of phosphorylation of adenylic acid is just that oxidoreduction which Rapkine [1938] has shown to be inhibited by O_2 [Meyerhof *et al.* 1937; Needham & Pillai, 1937]. Nevertheless, rapid changes between the reduced and oxidized state may permit Robison ester formation in the former and oxidative breakdown of the compound in the latter state (cf. the correlation of oxidation and phosphorylation in haemolysed blood [Runnström & Michaelis, 1935]). There is also the possibility of phosphorylation of glycogen by creatine phosphate via adenylypyrophosphate [Lehmann & Needham, 1937]. Sacks & Sacks [1933] found that "hexosephosphate" was formed in intact muscle only at the expense of creatine phosphate breakdown. Yet Sacks [1933; 1938] doubts whether hexosephosphate is formed at all in the "steady state" in aerobic muscle.

An increase of aerobic fermentation of yeast by cysteine and SH glutathione has been described [Quastel & Wheatley, 1932; Runnström & Sperber, 1937; 1938], although it has not yet been shown that this effect was due to an increase in Robison ester formation from glucose.

The action of reduced compounds on Robison ester formation throws new light on the work of Gaddie & Stewart [1935]. They observed an increased lactic acid formation from glycogen in muscle extracts after addition of SH glutathione. That other authors could not find the same for glycogen breakdown [Geiger, 1935] or phosphorylated glucose breakdown (muscle extract plus yeast hexokinase [Meyerhof & Kiessling, 1935]) may find its explanation in the possibility that Gaddie & Stewart were working with an oxidized extract, the others with sufficiently reduced enzyme solutions. Our finding may be thought to have a bearing on the question whether glutathione is a coenzyme for glycolysis in embryo. Needham & Nowinski [1937] and Needham & Lehmann [1937] suggest that the glycolytic breakdown in embryo may be different from that of glucose in yeast or glycogen in muscle. It has been pointed out that their arguments are negative and as such unconvincing in themselves [MacFarlane, 1939]. One of the positive arguments was that dialysed embryo enzyme needed glutathione for reactivation. This reactivation cannot be a revival of phosphorylation like the one described in this paper for rabbit muscle extract, as it is specific for glutathione and cannot be shown by using another SH compound like cysteine [Needham & Lehmann, 1937].

SUMMARY

1. Amylases inhibit the formation of Robison ester in muscle extracts. The hydrolytic enzyme acts simply as a competitor with the phosphorylating enzyme for the substrate, glycogen.

2. Oxidizing agents inhibit Robison ester formation: the agents used were catechol plus catechol oxidase, and SS-glutathione. Adrenaline in large amounts can be used instead of catechol. Small amounts of adrenaline had no effect on Robison ester formation.

3. Reducing compounds and agents increasing the content of SH-groups in the medium augment Robison ester formation. Such were SH-glutathione, cyanide, thiolacetic acid, denatured protein, sodium hydrosulphite and evacuation.

4. Muscle extract boiled for 2 min. is still able to phosphorylate glycogen and to form Robison ester.

5. Cu^{++} , Fe^{+++} , Fe^{++} and Zn^{++} inhibit Robison ester formation; Ni^{++} and Co^{++} increase it. Co^{++} increased Robison ester formation at optimum Mg^{++} concentration.

6. Ascorbic acid inhibits Robison ester formation. It acts in concentrations similar to those of glucose.

7. Insulin inhibits Robison ester formation but allows accumulation of Cori ester to go on. Its action seems largely to be due to the removal of Mg^{++} from solution. Insulin, the SS groups of which have been reduced either by cyanide or by muscle extract, does not inhibit Robison ester formation.

8. Data are given regarding the inhibition of phosphorylation by sugars. Of these the most active are glucose, mannose and Robison ester. They inhibit, like amylases, the phosphorylation of glycogen and consequently the formation of Robison ester. Hexosediphosphate increases phosphorylation of glycogen and consequently the formation of Robison ester. Its effect is not inhibited by iodoacetate or arsenate.

9. Points 1, 2 and 3 are discussed in their possible significance for the biochemistry of muscle.

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CXLI. HYDROLYSIS OF GLUTATHIONE BY BLOOD SERUM

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THE hydrolytic breakdown of oxidized glutathione (GSSG) by serum from cancer patients was reported recently by Waldschmidt-Leitz [1938]. It was implied that this observation might form the basis of a diagnostic test for cancer, since no activity was found with serum from apparently normal individuals. That hydrolysis occurred was deduced from the increase in free amino-groups observed in GSSG-cancer serum mixtures after incubation. Antiglyoxalase was assumed to be responsible for the cleavage.

Antiglyoxalase, an enzyme (or enzyme system) which destroys the ability of reduced glutathione (GSH) to activate glyoxalase, has been studied extensively in this laboratory [Platt & Schroeder, 1934; Woodward *et al.* 1935; Schroeder *et al.* 1935; Schroeder & Woodward, 1937]. The enzyme was found only in kidney and pancreas extracts, the former containing by far the larger amount. Studies on kidney extracts showed that the enzyme acts by bringing about hydrolysis of both peptide linkages of the GSH molecule, with liberation of the middle amino-acid, cysteine. The oxidized form, GSSG, was attacked similarly, with liberation of cystine.

In view of the possibility that the observation of Waldschmidt-Leitz might be of diagnostic value in cancer, it appeared desirable to investigate further the action of serum on glutathione. In our previous antiglyoxalase work use was made of two highly specific tests to demonstrate glutathione hydrolysis. One of these consisted in incubating GSH with the enzyme for a definite period under standard conditions, then measuring the unchanged GSH by means of the specific glyoxalase method of Woodward [1935]. Any loss of glyoxalase-activating power indicated hydrolysis of the tripeptide. The other test involved the measurement by the Sullivan [1929] method of cysteine or cystine formation in incubated enzyme-glutathione mixtures. Application of these two tests to serum-glutathione mixtures should give a clearer picture of any hydrolytic changes in the glutathione molecule than was provided by amino-acid titration data, and should answer the following questions: (1) Does serum bring about true hydrolysis of glutathione, or is the observed $-\text{NH}_2$ increase due to other factors? (2) If hydrolysis of glutathione actually occurs are both peptide linkages attacked, i.e. is cysteine liberated? (3) Can any differences be observed by these specific methods between the actions of normal and of cancer serum on glutathione?

Since GSH is partially converted into GSSG in the presence of serum, even under anaerobic conditions, use of GSH would involve a lack of uniformity of substrate. For this reason oxidized glutathione, the form used by Waldschmidt-Leitz, was chosen for the present work. In order to apply the glyoxalase method to the measurement of GSSG hydrolysis, it was necessary to reduce to GSH any GSSG remaining after the action of the serum, since this method measures only

GSH. The electrolytic procedure recently developed in this laboratory by Dohan & Woodward [1939] for reducing GSSG to GSH made this measurement possible.

The results obtained in the present investigation show that the ability of serum to hydrolyse GSSG has no value as a diagnostic test for cancer. True hydrolysis of the intact GSSG molecule was produced by all cancer blood sera, but, in disagreement with Waldschmidt-Leitz, hydrolysis of GSSG by normal sera also was observed. There was wide variation between the activities of different sera in each group, but the range of variation was the same for normal as for cancer sera. Other pathological sera likewise produced similar amounts of GSSG cleavage. Cystine was established as a final product of the GSSG hydrolysis, showing that all peptide linkages had been attacked. The enzyme in serum responsible for the hydrolysis is therefore similar to antiglyoxalase from kidney. The average amount found in serum is very small, however, compared with that in rat kidney, being in a ratio of about 1 : 1600.

Method

GSSG was incubated with blood serum in the proportion of 1 mg. GSSG to 1 ml. serum. To 4 ml. serum in a test tube were added 2 ml. 0.2 % GSSG (4 mg.). The tube was stoppered by a cotton-wool plug and placed in a water bath or incubator at 38° for 24 hr. At the end of this time 2 ml. of 0.8 *M* salicylsulphonic acid were added to stop the reaction and precipitate the protein. Free salicylsulphonic acid remained in the filtrate to the extent of approximately 2.5 %, a concentration desirable for the subsequent reduction.

The electrolytic reduction procedure of Dohan & Woodward [1939] was applied to 4–5 ml. of the filtrate, whereby GSSG is reduced to GSH and cystine is reduced to cysteine. The amount of GSH in the reduced filtrate was determined in 0.2 ml. portions by the manometric glyoxalase method of Woodward [1935]. Iodimetric titration could not be used for the GSH analysis since it does not distinguish between the intact GSH tripeptide and the SH-compounds which might be split from it. Cysteine analyses were carried out by the Sullivan method [1929] on either 1 ml. of the electrolytically reduced filtrate with 1/5 quantities of his reagents, or 2.5 ml. with 1/2 quantities.

If the serum showed more than a trace of haemolysis, correction for the possible cystine already present was made by a separate blank determination. Since serum itself contains no GSSG or cystine, a blank determination was not otherwise necessary. Most of the sera were used within 7 days, although samples were kept for much longer periods (71 days) with no change in activity.

Results

Tables I–III present the data obtained on the destruction of GSSG by serum and the resulting cystine formation. The destruction of GSSG was found to be from 16 to 99% with 10 normal sera, 11 to 81% with 10 cancer sera, and 11 to 72% with sera of 10 individuals with other pathological conditions. There is wide variation within each group, and each group covers approximately the same range. The conclusion is, therefore, that there is no correlation between the condition of an individual, diseased or otherwise, and the ability of his serum to destroy GSSG.

The amount of cystine formation resulting from the hydrolysis of the GSSG leads to the same conclusion. In general, the cystine formation parallels the GSSG loss. Except with a few sera which cause the highest amounts of GSSG destruction, the amount of cystine found entirely accounts for the loss of GSSG.

Table I. *Normal individuals*

Subject	Cleavage of 1 mg. GSSG by 1 ml. serum	
	GSSG lost %	Cystine formed %
1	99	62
2	97	61
3	74	46
4	56	51
5	30	33
6	28	36
7	24	29
8	19	25
9	17	—
10	16	16

Table II. *Cancer cases*

Case	Cleavage of 1 mg. GSSG by 1 ml. serum	
	GSSG lost %	Cystine formed %
1. Multiple carcinoma of pelvis and spine	81	69
2. Adenocarcinoma of breast—after operation and radiotherapy	53	45
3. Carcinoma of breast and lung—after radiotherapy	49	46
4. Carcinoma of breast, recurrent, with metastases—after radiotherapy	41	38
5. Lymphosarcoma of abdomen, untreated	44	41
6. Reticulum cell sarcoma, multiple	38	44
7. Multiple fibrosarcomata	37	43
8. Carcinoma of breast removed by operation 2 yr. previously. No recurrence apparent	19	32
9. Carcinoma of appendix with metastases—1 day after exploratory operation	18	31
10. Carcinoma of stomach—4 days after exploratory operation	11	24

Table III. *Miscellaneous pathological cases*

Case	Cleavage of 1 mg. GSSG by 1 ml. serum	
	GSSG lost %	Cystine formed %
1. Splenomyelogenous leukemia	72	63
2. Hypertensive arteriosclerosis with cerebral haemorrhage	60	59
3. Hypertensive arteriosclerosis	60	57
4. Hypopituitarism	51	39
5. Phlebosclerosis and atherosclerosis	54	52
6. Hypertension and atherosclerosis	36	26
7. Hypertension and atherosclerosis	36	27
8. Diabetes, insulin treated	25	35
9. Arteriosclerosis	21	21
10. Marked hypertension and arteriosclerosis	17	24
	13	23
	11	28

In cases where the cystine formation appears greater than the GSSG loss, explanation may be found in the tendency of the Sullivan method to yield values which are too high when the cysteine concentration of the solution being analysed is very low. In all cases cystine was an end-product of the hydrolysis of GSSG by serum.

Reduced glutathione, incubated 24 hr. with blood serum anaerobically in N_2 or in H_2 , was similarly hydrolysed. Considerable amounts of cysteine were found in unreduced filtrates from the incubation mixtures. The experiments were not of a quantitative nature, however, since there was always considerable loss of GSH and cysteine by oxidation.

SUMMARY

Blood serum was found to contain a small amount of an enzyme which hydrolyses oxidized glutathione with liberation of cystine. This hydrolysis is similar to that produced by antiglyoxalase from kidney, but the activity of serum is exceedingly small compared with that of kidney. The degree of hydrolytic activity of serum towards oxidized glutathione cannot be used as a diagnostic test for cancer, since there was no significant difference between the action of the normal and the cancer or other pathological sera examined.

The author wishes to acknowledge her indebtedness to Dr Ellice McDonald, Director, for his interest and support, and to Dr V. W. Murray Wright, Chief of Clinical Research of this laboratory and Chief of the Cancer Service of the Jewish Hospital of Philadelphia, for supplying the cancer blood samples used in this study.

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CXLII. THE NATURE OF PAPAIN

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THE widely accepted view that active papain contains SH groups, its oxidation to the disulphide form leading to its inactivation [Bersin, 1933; Purr, 1935] appears to be untenable in the light of more recent work. The irreversible inactivation of papain by iodoacetic acid, even in low concentrations, cannot be satisfactorily explained on the basis of its thiol nature, whilst the inhibition of its action on hippurylamide or on carbobenzyloxyisoglutamine by phenylhydrazine and acceleration of the action on albumin peptone by the same reagent, observed by Bergmann & Ross [1936] cannot be interpreted on simple oxidation-reduction considerations. In fact, Okumura [1938] working with purified papain preparations regards papain as a "special" protein containing an aldehyde group but neither a SH or SS group.

Bergmann's researches on papain [cf. Bergmann & Ross, 1936], employing synthetic substrates, led him to the view that its behaviour can best be explained on the basis that papain is a two-enzyme system, a view which he subsequently considered superfluous. Frankel *et al.* [1937], however, employing the fresh latex of *Carica papaya* as a source of the enzyme, adduced evidence to show that gelatin cleavage and peptone cleavage were two distinct processes. It thus appears that both the thiol nature of papain and its homogeneity have been questioned.

For reasons already mentioned in our earlier paper [Ganapathy & Sastri, 1938, 1], it is imperative for any work on the nature of papain to employ preparations obtained from freshly tapped latex of *C. papaya*. The results reported in this paper, obtained with such preparations, throw some light on the complexity of the enzyme. A preliminary communication on the subject appeared in *Nature* [Ganapathy & Sastri, 1938, 2].

EXPERIMENTAL

The papain preparations employed in this study were all obtained from the fresh latex of *C. papaya* grown in the Institute nursery.

SH-papain. Fresh latex (100 g.) was stirred up vigorously with 5 times its weight of water and filtered. The enzyme was precipitated from the filtrate by the addition of 5 vol. absolute alcohol. The precipitate was centrifuged, dissolved in water, filtered and the enzyme precipitated by the addition of alcohol, the process being repeated 3 times. The final precipitate was washed on the centrifuge with absolute alcohol and dried over CaCl_2 in a vacuum desiccator. The extractions and precipitations were carried out in the cold room at 0° (yield 11 %).

SS-papain. Fresh latex (100 g.) was stirred up with 5 times its weight of water and 2 ml. of H_2O_2 (Merck's 30 %) for about 1 hr. The supernatant fluid, at this stage, failed to give any test with nitroprusside. The precipitate obtained

by adding 5 vol. absolute alcohol to the clear filtrate, was separated by centrifuging, dissolved in water, treated again with 2 ml. H_2O_2 , filtered and the enzyme precipitated by the addition of alcohol, the process being repeated 3 times. The final precipitate was washed on the centrifuge with absolute alcohol and dried over $CaCl_2$ *in vacuo* (yield 12 %).

SS-papain preparations were also obtained by employing alloxan as the oxidizing agent. The procedure was essentially similar, except that the latex extract and alloxan were kept at room temperature (25–27°) for 12 hr. before precipitating the enzyme by alcohol. The red colour developed during this treatment could be eliminated by means of decolorizing carbon.

Four different preparations were obtained from samples of latex tapped on different days. The substrates employed were gelatin (gold label), egg albumin, Witte's peptone, Roche peptone, silk peptone and hippurylamide. The HCN activations were carried out according to the method of Willstätter & Grassmann [1924].

The reaction mixture consisted of 5 ml. of 5 % enzyme solution, 5 ml. of substrate (5 or 10 %), 5 ml. of McIlvaine's citrate-phosphate buffer (twice the usual concentrations) pH 3.8, 5 ml. of the activator (or water), to make up the total volume to 20 ml. The activity was determined by pipetting out 2 ml. of the reaction mixture after 24 hr. incubation at 38° into 2 ml. of 40 % formaldehyde and titrating against 0.1 N alkali with phenolphthalein as indicator. The figures in the following tables indicate increase in formaldehyde titration (ml. of 0.1 N alkali).

Table I. *SH-papain*

Substrate (10 %)	ml. of 0.1 N NaOH			
	Prep. I	Prep. II	Prep. III	Prep. IV
Gelatin without HCN	0.76	0.67	1.04	0.70
Gelatin with HCN	1.35	1.35	1.08	1.19
Peptone without HCN	1.12	1.10	1.18	1.27
Peptone with HCN	1.16	1.17	1.34	1.35

Table II. *SS-papain*

Substrate (5 %)	ml. of 0.1 N NaOH					
	Prep. I	Prep. II	Prep. III	Prep. IV	Prep. V*	Prep. VI*
Gelatin without HCN	0.25	0.18	0.36	0.34	0.60	0.52
Gelatin with HCN	0.48	0.30	0.81	0.80	—	—
Peptone without HCN	0.00	0.00	0.01	0.01	0.08	0.05
Peptone with HCN	0.40	0.31	0.50	0.48	—	—

* Preparations V and VI were alloxan-treated.

Table III. *SS-papain with different buffers*

Substrate 5 %	Without buffer	Acetic acid-acetate	Phosphate-citrate
Gelatin	0.19	0.25	0.27
Peptone	0.00	0.01	0.02

It will be seen from Table II that the SS-preparation, strikingly enough, hydrolyses gelatin but not peptone unless activated by HCN. It may be mentioned here that the reaction mixtures, except when treated with cyanide, failed to give the test with nitroprusside both before and after the reaction. Activation can be effected by H_2S , cysteine or glutathione. Identical results were obtained

with both citrate and acetate buffers, showing that the results are not influenced by the presence of buffer salts (Table III).

The results obtained with different substrates are given in Table IV.

Table IV. *Action on various substrates*

Substrate*	SS-papain		SH-papain	
	Without HCN	With HCN	Without HCN	With HCN
Gelatin	0.36	0.85	0.78	1.01
Egg albumin	0.24	0.25	0.26	0.28
Witte's peptone	0.01	0.48	1.16	1.18
Silk peptone	0.02	0.44	0.66	0.70
Roche peptone	0.01	0.46	0.44	0.46
Hippurylamide	0.00	0.40	0.46	0.56

* For SS-papain the substrate concentration was 5% while for SH-papain it was 10%.

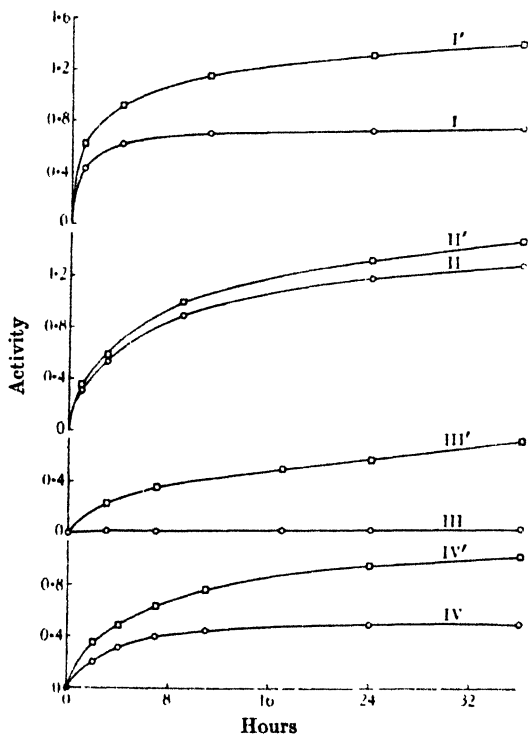


Fig. 1.

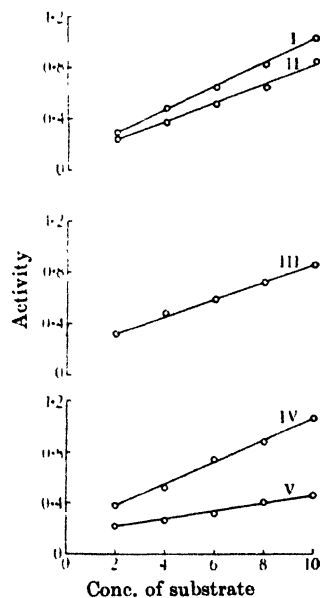


Fig. 2.

Fig. 1. Hydrolysis of gelatin and peptone by SH- and SS-papain. I, SH-papain plus gelatin; II, SH-papain plus peptone; III, SS-papain plus peptone; IV, SS-papain plus gelatin; I', II', III', IV', corresponding activities in the presence of cyanide.

Fig. 2. Influence of substrate concentration on the rate of hydrolysis of gelatin and peptone by SH- and SS-papain. I, Natural papain plus gelatin; II, natural papain plus peptone; III, SS-papain plus cyanide plus peptone; IV, SS-papain plus cyanide plus gelatin; V, SS-papain plus gelatin.

SS-papain hydrolyses both egg albumin as well as gelatin but has no action on peptones or hippurylamide.

The courses of hydrolysis of gelatin and peptone by SH- and SS-papain preparations are indicated in Fig. 1. Natural papain, i.e. a filtered aqueous extract of the fresh latex, which contains a large concentration of sulphhydryl compounds behaves exactly like the precipitated SH-preparation, in that there is only a slight increase in the degree of hydrolysis in the presence of cyanide.

Fig. 2 shows the variations in the hydrolysis of gelatin and peptone by SH- and SS-papains, with increase in the substrate concentration. The relationship is more or less linear.

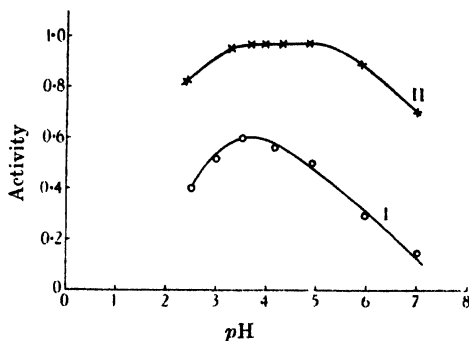


Fig. 3. Influence of pH on the hydrolysis of gelatin by SH- and SS-papain; I, SS-papain; II, SH-papain.

Fig. 3 shows the influence of pH on the hydrolysis of gelatin by SH- and SS-papain preparations. The optimum activity in the case of SH-papain is spread over the range pH 3-5. The SS-preparation, has a sharp optimum at pH 3.6-3.8.

The influence of added substances. Table V shows the influence of adding maleic acid, glutathione, iodoacetic acid and HCN on the hydrolyses of gelatin and peptone by SH- and SS-papain preparations.

Table V

Activator	SH-papain		SS-papain	
	Gelatin	Peptone	Gelatin	Peptone
Unactivated	0.47	0.56	0.27	0.00
HCN (1%)	0.68	0.58	0.46	0.40
Glutathione 20 mg.	0.70	0.61	0.50	0.44
Maleic acid 15 mg.	0.31	0.08	0.27	0.00
Iodoacetic acid 0.1 mg.	0.00	0.00	0.00	0.00

DISCUSSION

The oxidized preparations which give negative nitroprusside tests, possess considerable activity towards gelatin while being without effect on peptone. This observation warrants the conclusion that for "gelatinase" activity of papain the SH group is not essential. The "gelatinase" activity of the SS-papain preparation corresponds to about 60% of the "full" (i.e. HCN-activated) activity.

It has been shown by Hopkins *et al.* [1938] that those enzymes which depend for their activity on the presence of a SH group are specifically inactivated by maleic acid. The figures given in Table V show that while "gelatinase" activity is not affected by the addition of maleic acid, the "peptonase" activity of the SH-preparation is inhibited. This further supports the view that

the SH group is not essential for "gelatinase" action; it appears to be necessary for "peptonase" action.

Iodoacetic acid inactivates both SH- and SS-preparations; the inactivation is irreversible and the amount required for this inactivation is far less than is necessary for reacting with the SH groups. This shows that both for gelatinase and peptonase activities some group (or groups) other than SH is essential.

SUMMARY

1. Preparations of papain (SS-papain) obtained by completely oxidizing the SH groups of natural papain by H_2O_2 or alloxan, retain the property of hydrolysing gelatin or egg albumin. They are however inactive towards peptones.

2. The optimum pH for gelatin hydrolysis by SS-papain is 3.6-3.8; that for hydrolysis by SH-papain is spread over the range pH 3-5.

3. Cyanide and glutathione activate SS-papain; this activation extends the range of action of the enzyme, which is then capable of hydrolysing peptones. Maleic acid is without effect on gelatinase; it inhibits the hydrolysis of peptone by SH-papain. Iodoacetic acid inactivates irreversibly both SH- and SS-papain preparations.

4. The SH group is not essential for gelatinase activity; it appears, however, to be necessary for peptonase activity. Another group which reacts with iodoacetic acid irreversibly appears to be essential for both the gelatinase and peptonase activities.

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CXLIII. A SIMPLIFIED METHOD FOR THE ISOLATION OF GLUTATHIONE FROM YEAST

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ONE of the difficulties in isolating glutathione from yeast is the extremely slow filtration encountered in removing the yeast residues after extraction. For example, in Pirie's [1930] method, which is probably the simplest thus far proposed, the filtration requires 4-6 hr. It has now been found that this time can be reduced to 20 min. by liberating the glutathione with acetone, extracting the yeast residue with water, and filtering in the presence of a large quantity of infusorial earth. The details of the method are as follows.

EXPERIMENTAL

Two lb. (900 g.) of pressed baker's yeast are crumbled coarsely by hand, dropped into 2500 ml. of acetone and stirred for 10 min. The yeast residue is filtered on an 8 in. Büchner funnel and washed once with a small quantity of acetone, suspended in about 1350 ml. of water and stirred for a few minutes, until homogeneous, with a strong electric stirrer; the larger clumps may be broken up by hand if necessary; 200 g. of infusorial earth are added, and the stirring is continued for a few minutes longer; the mixture is then filtered through a 12 in. Büchner funnel, about 1150 ml. of clear, light-yellow filtrate being obtained in 20 min.

The remainder of the procedure is essentially that described by Hopkins [1929]. The filtrate is made approximately 0.5*N* with respect to H_2SO_4 by addition of $\frac{1}{3}$ vol. 5*N* acid, and warmed to 50°. A suspension of 0.25-0.30 g. of Cu_2O in about 20 ml. of water is added in 2 ml. portions, with stirring.¹ The white cuprous glutathione formed is allowed to settle for 30 min. and, after decantation of the supernatant liquid, is washed by centrifuging until sulphate-free (usually 5 washings with 100 ml. portions of O_2 -free water are sufficient). It is suspended in 35-40 ml. of water and treated with H_2S for 5-10 min. The cuprous sulphide formed is removed by centrifuging and filtering. After expulsion of the excess H_2S with a stream of nitrogen, the filtrate is transferred to a 3 in. crystallizing dish and evaporated to dryness by placing it overnight in a vacuum desiccator containing H_2SO_4 . The clear glassy residue thus obtained is dissolved by slight warming, in about 7 ml. of water, and mixed with 3 or

¹ If desired, the cuprous oxide may be prepared immediately before use by heating 50-60 ml. of Benedict's qualitative sugar reagent (173 g. sodium citrate, 100 g. anhydrous Na_2CO_3 and 17.3 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, made up to 1 l. with water) with excess glucose for a few minutes at 80-90° until the scarlet-coloured oxide appears. The hot suspension thus obtained is added directly, in small portions, to the yeast extract; 1 ml. of the reagent yields approximately 5 mg. of cuprous oxide. When this method is used, it is advisable first to add 20 or 25 ml. extra 5 *N* H_2SO_4 to the yeast extract to neutralize the alkali in the Benedict's reagent.

4 ml. of alcohol, a little more alcohol being poured over the surface of the solution. After seeding, the dish is placed over NaOH in a vacuum desiccator containing also a small beaker of alcohol. Crystallization begins in 2 or 3 hr., and is allowed to proceed overnight. The dry crystalline residue is transferred to a small funnel by means of 70 % alcohol, and washed first with 70 % and finally with absolute alcohol. It is dried over H_2SO_4 .

The yields have ranged from 0.5 to 0.75 g., depending upon the original glutathione content of the yeast. Pirie [1930] reported somewhat higher yields (0.9 g. from 2 lb. of yeast); he, however, extracted his yeast residues three times instead of once. The purity of the product obtained as just described is sufficiently high for most purposes (98 %); found, for two different preparations, 10.68 and 10.27 % sulphur; theory, 10.42 %. Samples of three different preparations, titrated by the method of Woodward & Fry [1935], required per mg. 3.28, 3.26 and 3.32 ml. of 0.001 *N* iodate; theory, 3.26 ml. After one recrystallization from dilute alcohol the sulphur content was 10.43 %.

The cuprous glutathione precipitated in this procedure is impure, probably containing substances derived from the infusorial earth. The sulphate-free salt, after being washed with alcohol and dried to constant weight at 50° *in vacuo*, contains 15–16 % Cu (electrolytic determination) instead of the 17.2 % required by theory. This, however, causes no difficulty, since the impurities are removed in the subsequent H_2S treatment.

By this new method it is possible to complete precipitation of the cuprous glutathione within 1 hr., starting from the original yeast. When larger amounts of glutathione are needed, it is advantageous to carry several 2 lb. lots of yeast to this stage, the precipitates being combined before washing.

The yield of final product obtained represents about 50 % of the glutathione present in the original yeast, as shown by the specific glyoxalase method of Woodward [1935]. For example, a 2 lb. lot of pressed yeast contained 1.37 g. of reduced glutathione. The filtrate obtained on extraction (after acetone treatment, which liberates all the glutathione but removes none) contained 1.09 g., or 80 % of the total. About 80 % of this was precipitated as the cuprous salt, and the yield of crystalline glutathione obtained from the cuprous salt was also 80 %.

The acetone may be recovered by simple distillation. Extraction of yeast with aqueous acetone (60 %), as was done by Kozlowski [1931], yielded filtrates from which cuprous glutathione could not be precipitated directly in satisfactory form. Extracts prepared by first liquefying the yeast with a small quantity of acetone (100 ml./lb.), then extracting with water, contained little glutathione.

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CXLIV. THE PREPARATION OF PHENYL PHOSPHORIC ESTERS

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THE phosphoric esters of simple phenols have been prepared through the action of phosphorus oxychloride on the phenol. Thus Rapp [1884] and Jacobsen [1875] prepared monophenyl phosphoryl chloride; monophenyl phosphoric acid was prepared by Iwatsura [1926] and the phosphoric esters of several phenols by Asakawa [1929]. A method for making disodium phenyl phosphate was recently described by Freeman & Colver [1938]. The procedures used by these authors have been essentially the same, i.e. a prolonged refluxing of phosphorus oxychloride with the phenol at a high temperature. The final reaction mixture consists of unchanged phosphorus oxychloride and the mono-, di- and tri-esterification products. These are separated by fractional distillation under diminished pressure. The phenyl phosphoryl chlorides are decomposed with water to give the phosphoric acid esters.

The introduction of phenyl phosphoric ester for the study of phosphatase activity in blood serum [King & Armstrong, 1934] and in milk [Kay & Graham, 1935], and the use of phosphoric esters of several phenols for studying the kinetics of phosphatase hydrolysis [King & Delory, 1938; 1939] have made desirable the description of a simple procedure for preparing phenyl phosphoric esters. The reaction of phenols with phosphorus oxychloride in pyridine solution takes place quickly and without the necessity for prolonged heating. When the phenol and the oxychloride are mixed in molecular proportions there is only one product of the reaction, namely the primary ester, and this is usually uncontaminated by unesterified phosphoric acid. The separation of the free ester from ether solution, or of its salts from aqueous alcohol is easily accomplished. The following esters have been prepared by this method: phenyl phosphate, *o*-methylphenyl phosphate, *p*-bromophenyl phosphate, *p*-nitrophenylphosphate and cyclohexanol phosphate.

EXPERIMENTAL

Barium phenyl phosphate

To 10 ml. of phosphorus oxychloride (17.1 g.) in a 1 l. beaker is added, slowly and with stirring, a solution of 10.5 g. of phenol in 50 ml. of dry pyridine. The mixture becomes hot and a crystalline precipitate of pyridine hydrochloride separates. The beaker is covered with a watch glass and the mixture is boiled gently for 10 min. The hot solution solidifies on cooling. The phenyl phosphoryl chloride is decomposed with water (5 ml.) added dropwise and slowly. It is advisable to cool the beaker under the cold water tap during this process, as the reaction may be somewhat violent and much heat is developed. Hot saturated aqueous barium hydroxide is added until the mixture is pink to phenolphthalein. The barium salt of phenyl phosphoric ester separates as a heavy flocculent precipitate. This is increased by adding alcohol, with stirring, to the capacity of the beaker and leaving in the ice chest overnight. The barium phenyl phosphate

is filtered by suction, washed first with 50 %, then with absolute alcohol, and is dried *in vacuo* over H_2SO_4 . Yield of crude barium salt 29.4 g. and 30.2 g. in two typical experiments (85 and 87 % of theoretical). Crystallized from hot aqueous alcohol barium phenyl phosphate separates as small glancing platelets. (Found: P, 8.95, 8.97%; Ba, 40.4, 40.0%. Calc. for $\text{BaC}_6\text{H}_5\text{PO}_4$, $2\text{H}_2\text{O}$: P, 8.98; Ba, 39.9 %.)

Disodium phenyl phosphate

This salt is generally used for the phosphatase determinations and for other enzymic studies. For that reason its preparation is described. 10 g. of the Ba salt are dissolved in 300 ml. of 0.2 *N* HCl, and the solution shaken out with 6 changes of 100 ml. of ether. The combined ether extracts are diluted with half their volume of alcohol and 40 % aqueous NaOH added with stirring until the mixture is alkaline. The precipitate of disodium phenyl phosphate is filtered, washed with alcohol and dried *in vacuo* over H_2SO_4 .

o-Methylphenyl phosphate

5 g. of *o*-cresol in 25 ml. of pyridine were added to 4.15 ml. of phosphorus oxychloride. The reaction mixture was heated for 1 hr., cooled and diluted slowly with water to a total volume of 100 ml. The solution was neutralized with 40 % NaOH. 9.5 g. of barium chloride (a 50 % excess over the theoretical), dissolved in 50 ml. of water, were added with stirring, followed by 2 vol. alcohol. The heavy precipitate which separated on standing was filtered, and extracted with hot water (500 ml.). On adding 2 vol. alcohol to the filtered extract, the barium *o*-methylphenyl phosphate separated as a precipitate of small plate-like crystals. Analysis: P, 8.97 %; Ba, 40.2 %. Calc. for $\text{C}_7\text{H}_7\text{O}_4\text{PBa}$, H_2O : P, 9.09 %; Ba, 40.2 %.

p-Bromophenyl phosphate

A solution of 17.3 g. of *p*-bromophenol in 50 ml. of pyridine was added slowly and with stirring to 9 ml. of phosphorus oxychloride. The mixture was allowed to cool spontaneously and deposited crystals of pyridine hydrochloride. Water was added dropwise, until the initial violent reaction had subsided, and then in amount sufficient to make a final volume of about 150 ml. Hot saturated aqueous barium hydroxide was added until the mixture was alkaline, followed by an equal volume of alcohol. The barium salt of *p*-bromophenyl phosphate separated as a sheeny white crystalline precipitate. (Found: P, 7.69 %; Ba, 36.6 %. Recrystallized from aqueous alcohol the product analysed: P, 8.02, 7.98 %; Ba, 35.2, 35.8 %. Calc. for $\text{C}_6\text{H}_4\text{O}_4\text{PBrBa}$: P, 7.99 %; Ba, 35.3 %.)

p-Nitrophenyl phosphate

13.9 g. of *p*-nitrophenol were dissolved in 50 ml. of pyridine and added to 9 ml. of phosphorus oxychloride. The mixture was diluted with water to a final volume of 150 ml. Hot saturated barium hydroxide and alcohol were added as in the case of the bromophenyl phosphate. The barium salt was obtained as pale yellow plate-like crystals. (Found: P, 7.97, 7.99 %. Calc. for $\text{C}_6\text{H}_4\text{O}_6\text{NPBa}$, $2\text{H}_2\text{O}$: P, 7.95 %.)

cycloHexanol phosphate

5.7 ml. of cyclohexanol were added to 5 ml. of phosphorus oxychloride in 50 ml. of pyridine. The product was treated with water, diluted with aqueous alcohol and precipitated with barium hydroxide. The crude barium salt was only slightly soluble in water. It was shaken with alcoholic *N* H_2SO_4 in amount

equivalent to the barium present (determined by analysis). A small excess of H_2SO_4 in the filtrate was removed with benzidine, and the *cyclohexanol* phosphate precipitated with alcoholic potassium hydroxide. (Found: P, 11.9%; K, 31.1%. Calc. for $\text{K}_2\text{C}_6\text{H}_{10}\text{PO}_4$: P, 12.1%; K, 30.6%.)

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CXLV. THE RATES OF ENZYMIC HYDROLYSIS OF PHOSPHORIC ESTERS

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THE markedly different rates at which esters of phosphoric acid are hydrolysed by both animal and vegetable phosphatases have been noted by several workers, e.g. Martland *et al.* [1924], Kay [1926], Roche [1931] and Asakawa [1929]. Most primary esters of phosphoric acid are optimally hydrolysed by animal phosphatases at an alkaline reaction, but both the *pH* optimum and the rates of hydrolysis with the same enzyme preparation differ greatly with different esters. This is the case even with such closely related substances as α - and β -glycerophosphate [cf. Rae *et al.* 1934]. With more pronounced variations in the nature of the esterified alcohol or phenol the differences become more marked: thus phenyl phosphate is split by bone phosphatase more than twice as fast as glycerophosphate and many times faster than ethyl phosphate [King & Armstrong, 1934]. The *pH* optima for secondary esters, all near neutrality, do not differ so greatly as in the case of primary esters, but the rates of hydrolysis show the same large variations.

With more complex phosphoric esters, i.e. the phospholipins, it was found [King, 1934] that similar changes could be brought about in *pH* optimum and rate of hydrolysis by introducing changes in the structure of the esterified grouping. Lysolecithin was hydrolysed by the enzymes of the intestinal mucosa and of bone at a much faster rate than the parent lecithin and optimally at a more alkaline reaction. By brominating lecithin the rate of enzymic hydrolysis was raised 10 to 100 times, and the optimum *pH* of hydrolysis rose from 7.4 to 8.4. An explanation was sought in the nature of the substituent groups of the alcohol residue, and the suggestion was advanced that the more negative these groupings the faster would be the rate of hydrolysis and the more alkaline the *pH* optimum. This hypothesis that the negativity of the alcohol part of the ester influences the rate of enzymic decomposition is tested further in the present study.

A comparison is made of the rates of hydrolysis of several simple phosphoric esters in relation to the degree of acidity of the ester and the optimum *pH* of hydrolysis. The following esters have been studied, ethyl, α - and β -glycero, cresyl, phenyl, nitrophenyl and bromophenyl phosphates.

EXPERIMENTAL

The experimental work fell naturally into three parts, determination of the titration curves and *pK* values, determination of the optimum *pH* values for enzymic hydrolysis, and comparison of the rates of hydrolysis of the various substrates each at its optimum *pH*.

The titration constants were determined, using the hydrogen electrode, and saturated calomel electrode, according to the general procedure followed by Pirie & Pinhey [1929]. The change of *pH* on addition of 0.1 *N* HCl to 20 ml. of the 0.01 *M* solution of the salt of the ester was plotted on a graph, and from this the *pK* value could be derived.

For the determination of the *pH* optima of hydrolysis it was necessary to choose a buffer capable of giving *pH* values from 7.9 to 11 in order to embrace the full range of *pH* optima covered by the esters. This was accomplished by using a combination of the Michaelis [1930] and Kolthoff [1925] buffers, i.e. a mixture of veronal, Na_2CO_3 and HCl . Each buffer contained the same amount of veronal, namely 25 ml. of 0.1 *M* sodium veronal per 100 ml., and the same amount of Na_2CO_3 . A range of *pH* 7.87–10.6 was obtained by including amounts of 0.1 *N* HCl varying from 2.5 to 40 ml.

The following technique was adopted to avoid loss of CO_2 during the preparation of the buffer: 25 ml. of 0.1 *M* sodium veronal were carefully pipetted into a 100 ml. graduated flask, so as to overlay the appropriate amount of 0.1 *N* HCl . 25 ml. of 0.1 *M* Na_2CO_3 were placed in the flask and the contents mixed by gentle rotation. Water was added to the mark and the contents of the flask thoroughly mixed.

The *pH* curve for this buffer system was determined with the hydrogen electrode¹ and the saturated calomel electrode both at 22° and at 37°. The standard of reference was a sodium acetate-acetic acid buffer of *pH* 4.63 [cf. Sendroy, 1938]. As a check, reference measurements were made on Michaelis veronal buffer mixtures, and Kolthoff mixtures. We were able to check the *pH* values recorded by these authors with a maximum difference of 1 mV. corresponding to *ca.* 0.02 *pH*.

The source of the phosphatase used in these experiments was a powder prepared from dogs' faeces by the method of Armstrong [1935]. This material was freely miscible in water and was of great potency. The amount of this phosphatase preparation necessary to liberate measurable amounts of phosphorus in the required time (5 min.) was so small and contained so little protein material as to give no clouding with the ordinary protein-precipitating reagents. This made unnecessary the usual use of trichloroacetic acid followed by filtration to remove precipitated protein material. It was necessary only to stop the enzyme action at the desired time, and this could be done by the addition of the acid molybdate reagent used for the colorimetric phosphate determination.

For each experiment, an amount of the phosphatase preparation was weighed and dissolved in water so that an activity of approximately 30 King-Armstrong [1934] units per 100 ml. was obtained. MgSO_4 was added in amount sufficient to give a concentration of 0.01 *M* [cf. Kay, 1930]. The phosphatase activity of this solution was then determined,² and an appropriate dilution made to give a final activity of 25 units per 100 ml. The diluted enzyme solution then contained 1 unit of phosphatase in 4 ml.

The substrate was prepared by dissolving the Na salt of the ester in water to make a 0.01 *M* solution. Ba salts of the esters were dissolved in water and treated with the requisite amount of H_2SO_4 ; the solution was neutralized with NaOH , made to volume, mixed and filtered. The strength of each substrate solution was confirmed by colorimetric analysis for total P by the perchloric acid digestion method [King, 1932].

¹ Since it was considered advisable to establish equilibrium as rapidly as possible the electrode described by Moloney [1921] was used.

² 0.2 ml. of the phosphatase solution, together with 4 ml. of *M*/200 disodium phenyl phosphate in *M*/20 sodium veronal, is incubated at 37° for 30 min. The enzyme action is stopped by the addition of 1.8 ml. of diluted (1 in 3) Folin-Ciocalteu [1927] phosphotungstic-phosphomolybdic acid reagent. 4 ml. of the mixture (filtered if necessary) are treated with 1 ml. of 25% Na_2CO_3 and the blue colour compared against an appropriate phenol standard. The increase in free phenol is taken as a measure of the phosphatase activity. A phosphatase unit is defined as the amount of enzyme necessary to liberate 1 mg. of phenol in 30 min., under the above conditions.

For the determination of the optimum *pH* for hydrolysis of each substrate, and the relative amount of hydrolysis, the following procedure was adopted. Into each of five 15 ml. flasks were pipetted 4 ml. (1 unit) of the enzyme preparation. The flasks were placed in a water bath at 37°. Five test tubes containing an appropriate range of buffers to cover the expected optimum *pH* of the substrate (determined by a rough preliminary experiment) were likewise placed in the water bath. A 4 ml. pipette was introduced into each tube of buffer ready for use. The substrate solution was also placed in the bath. When the temperature of the solutions to be used had reached 37° (2–4 min.) the experiment was started. 4 ml. of the buffers were pipetted into the flasks containing the 4 ml. of phosphatase. 2 ml. of the substrate were then added to each of the flasks by means of a graduated pipette. (It is advisable to have an observer standing by with a stop-watch to note the time when the substrate is added to each tube.) The contents of the flasks were rapidly mixed. After a lapse of 5 min., 2 ml. of 5% ammonium molybdate solution in 15% H_2SO_4 were added. This was pipetted into each flask at the same rate as the substrate had been previously added. The ester was thus in contact with the phosphatase exactly 5 min. in each flask before the destruction of the enzyme by the acid ammonium molybdate.

The flasks were immediately cooled and to each were added 0.5 ml. of aminonaphthol sulphonic acid solution and water to the 15 ml. mark. The blue colours thus obtained were read in a colorimeter against an appropriate standard.

The above procedure was used to determine the optimum *pH* of hydrolysis for each phosphoric ester. In Fig. 1 are shown four curves relating the degree

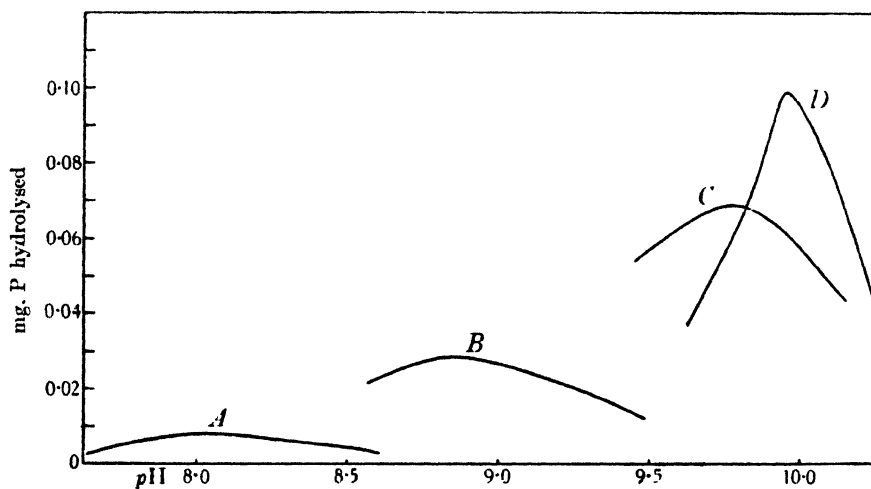


Fig. 1. Optimum *pH* for hydrolysis of phosphoric esters. *A*, ethyl phosphate; *B*, β -glycerophosphate; *C*, phenyl phosphate; *D*, bromophenyl phosphate. Substrate 0.002 *M*, phosphatase 1 unit, veronal-sodium carbonate buffer, 5 min. 37°.

of hydrolysis to the *pH*. The extent of the enzymic hydrolysis of the ester is expressed in terms of the actual mg. of P liberated in each test, i.e. the P set free by 1 unit of phosphatase in 5 min. The difference in the extents to which the esters are attacked is remarkable: ethyl phosphate is much more slowly split than glycerophosphate, and the introduction of a bromine atom has greatly increased the rate at which the phenyl phosphoric ester is split. The great

variation from one ester to another in the *pH* optimum is likewise seen. The figures determined for the *pH* optima for the several esters studied are contained in Table I.

Table I. *Rates of enzymic hydrolysis of phosphoric esters*

Substrate: phosphoric esters	<i>pK</i>	Optimum <i>pH</i> of hydrolysis	mg. P hydrolysed per phosphatase unit	Relative rate of hydrolysis (β -glycerophosphate = 1)
Bromophenyl	5.44	9.96	0.099	3.4
Nitrophenyl	5.70	9.80	0.082	2.8
Phenyl	5.73	9.76	0.069	2.4
<i>o</i> -Methylphenyl (<i>o</i> -cresyl)	6.04	9.55	0.042	1.5
β -Glycero	6.34	8.82	0.029	1.0
α -Glycero	6.44	8.62	0.019	0.7
Ethyl	6.45	8.08	0.008	0.3

The comparison of the relative rates of hydrolysis of the several esters was redetermined in an experiment in which all the esters were used simultaneously, each at its optimum *pH*. In this way the relative rates were determined with the identical enzyme solution and at the same time. This experiment was repeated several times, but with very little variation in the results obtained. The average figures are set out in Table I.

Table II. *Hydrolysis of phosphoric esters by several phosphatase preparations*

mg. P liberated by 1 ml. of phosphatase solution in 1 hr. at 37°

Substrate: phosphoric esters	Phosphatase preparation					
	Rabbit kidney	Pig kidney	Human intestine	Human kidney	Human bile	Dog faeces
Bromophenyl	0.19	0.24	0.99	0.78	1.37	3.3
Nitrophenyl	0.13	0.17	0.56	—	0.98	2.4
Phenyl	0.10	0.12	0.52	0.58	0.98	—
Glycero	0.05	—	—	—	0.30	1.7
Ethyl	0.02	—	—	—	0.25	0.58

Table III. *Hydrolysis of secondary phosphoric esters by phosphatase*

5 ml. 0.01 *M* NaR_2PO_4 + 5 ml. veronal buffer *pH* 7 + 0.5 ml. enzyme solution

	mg. phosphorus hydrolysed		
	24 hr.	48 hr.	72 hr.
Diphenyl phosphate			
Rat kidney	0.031	0.042	0.050
Cat kidney	0.009	0.024	0.059
Cat intestine	0.039	0.041	0.063
Rabbit intestine	0.083	0.113	0.136
Rat bone	—	0.007	—
Chicken bone	—	0.011	—
Dicresyl phosphate			
Rat kidney	0.003	0.013	0.019
Cat kidney	0.005	0.011	0.019
Cat intestine	0.010	0.023	0.027
Rabbit intestine	0.046	0.089	0.125
Diethyl phosphate			
Rat kidney	0.002	0.002	0.003
Cat kidney	0.004	0.006	0.008
Cat intestine	0.002	0.007	—
Rabbit intestine	0.006	0.014	—

The relative rates of hydrolysis of some of the esters have also been studied with other phosphatase preparations. The results for these experiments are contained in Table II, and are in substantial agreement with the findings obtained with the more highly purified phosphatase preparation. With the three secondary phosphoric esters which have been prepared the same phenomenon of different rates of hydrolysis is likewise seen. Diphenyl phosphate is attacked more quickly than dicresyl and dicresyl more quickly than diethyl phosphate. Some results for these esters are given in Table III.

DISCUSSION

All determinations of relative rates of hydrolysis for the different esters, and all pH activity curves, have been made with the same amount of enzyme and with the same final concentration of substrate, namely $0.002 M$ P ester. It is believed that the results are comparable amongst themselves, and that a true picture of relative velocities of hydrolysis is shown. Under the conditions of the experiments, i.e. 5 min. enzymic action at the optimum pH , it may be expected that the observed figures do not differ markedly from initial velocities.

Martland & Robison [1927] found no difference in the rates of hydrolysis of glycerophosphate between concentrations of 0.003 and $0.3 M$. At lower concentrations both Asakawa [1928] and Jacobsen [1933] found that the pH optimum of glycerophosphate varied with the substrate concentration. Folley & Kay [1935] found a pH optimum of 10 for phenyl phosphate hydrolysed by mammary phosphatase in glycine buffer when the concentration of ester was $0.0045 M$, while with a concentration of $0.00023 M$ the lower pH optimum of 9.3 was found. They concluded that the enzyme was fully combined with the ester at the higher substrate concentration but not at the much lower one represented by $0.0023 M$. The pH optimum of 9.8 found in this experiment for phenyl phosphate is slightly lower than that recorded by Folley & Kay (10.0), as likewise that for β -glycerophosphate, i.e. 8.8, as compared with the usually accepted value of 8.9 [cf. Kay, 1932]. It was thought possible that, with the very potent enzyme preparation used, higher pH optimum values might have been observed with greater concentrations of the substrate. In control experiments with phenyl phosphate and with α -glycerophosphate the same pH optima were observed at $0.002 M$ concentration of ester as at $0.02 M$, although there was a somewhat greater amount of total hydrolysis with the stronger substrate, i.e. with an increased substrate/enzyme ratio. With β -glycerophosphate the slightly higher value (pH 8.9) was found. It is not certain, of course, that the enzyme contained in the preparation from dog faeces is identical with the mammary gland phosphomonoesterase, or that the different buffers used were without effect. These aspects of the problem will be investigated further.

On the basis of the determined constants for the Michaelis-Menten [1913] equation, Folley & Kay pointed out that "the 'alkaline' phosphomonoesterase has a much greater affinity for the phenyl phosphate than for glycerophosphate, which accords well with the fact that the former substrate is hydrolysed by it more rapidly than the latter". The data here presented appear to support the hypothesis that the negativity of the esterified alcohol or phenol, as evidenced in the pK of the resulting substituted phosphoric acid, conditions the pH at which the enzyme optimally combines with the ester and the rate at which cleavage takes place. Although the Michaelis constants are not yet known for several of the esters used in the present investigation, the general behaviour fits well with the conception that the more strongly acid the phosphoric ester the greater will be the affinity of the enzyme for it.

SUMMARY

A study has been made of the rates of enzymic hydrolysis of several phosphoric esters. The rate of hydrolysis and the optimum pH for hydrolysis appear to increase with increasing acidity of the phosphoric ester.

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CXLVI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL¹

IV. THE RETENTION OF THE DRUG IN THE ANIMAL BODY

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IN previous papers [Dangerfield *et al.* 1938; Boursnell *et al.* 1939] a method for the determination of Bayer 205 was described, and observations were made on the persistence of this drug in the blood stream after injection into animals. The long retention of the drug in the body is of special interest in view of the prolonged protective effect against trypanosomiasis produced by the injection of Bayer 205, and it was considered desirable that further investigations should be carried out to determine the cause of this retention. The prophylactic value of this drug, for man and other animals, has been the subject of a large number of reports [for the literature, cf. Findlay, 1930, p. 271; 1939, p. 205], and most authorities agree that the injection of 1 or 2 g. will protect man against trypanosome infection for several weeks, and in some cases for several months. This long protective action has usually been explained by the assumption that there is retention of the drug by the body, and support for this view has been given in earlier papers of this series.

The molecule of Bayer 205 (mol. wt. 1428) is relatively large, but not sufficiently large to suggest that the drug will not be excreted by the kidney; indeed there is evidence that appreciable quantities are excreted in the urine during the first few days after injection. Some other explanation must therefore be sought to account for retention in the body. The experiments described in this paper have been carried out to determine (a) whether there is any appreciable storage of the drug in the liver, kidney and other organs and tissues, and (b) whether persistence in the blood stream is due to combination of the drug with plasma proteins.

EXPERIMENTAL

Bayer 205 determinations

On plasma, serum and protein precipitates. These determinations were carried out as described in previous papers [1938; 1939]. Distilled water was added to bring the volume to about 3.0 ml. before the addition of 3.0 ml. of conc. HCl. In most of these determinations "Analar" HCl has been used, after dilution to 10.4*N*, the strength of the conc. HCl hitherto employed; there is no suggestion, however, that the ordinary pure HCl is not suitable for this determination.

¹ This communication represents a third report to the Medical Research Council at whose request this work was undertaken.

All the measurements were made with the Lovibond tintometer discs previously described. Compensation for the small blank value for normal plasma or serum (equivalent to about 1.0 mg. Bayer 205/100 ml.) was effected by a control tube containing a corresponding amount of hydrolysed, diazotized and coupled normal plasma, serum or plasma-protein. The hydrolyses or diazotizations were usually carried out in duplicate and these almost invariably showed satisfactory agreement. The results recorded in the tables are average values for two or more determinations, and have been corrected, unless otherwise stated, for the blank values for normal serum, plasma etc.

On tissues. As soon as each rabbit was killed, various organs were removed,¹ dried on filter paper and weighed. Weighed amounts of the tissues (usually 1.5–2.5 g. except where the amount available was small) were quickly transferred to stoppered pyrex tubes graduated at 10 ml. Water was added to give a volume of about 3 ml., and 3 ml. of 10.4 *N* HCl were added at once to prevent bacterial or enzymic changes in the tissue. After hydrolysis many of these solutions had a very dark colour which was not completely removed by treatment with kaolin. To compensate for this colour, a control mixture was prepared for each hydrolysate, containing all the reagents used for the colorimetric determination except sodium nitrite, and when this control solution was placed in the appropriate part of the comparator, satisfactory matching of the colours could be made. For several reasons it was not possible to compensate for the blank values of normal tissues by the simple procedure used with plasma or serum, and it was necessary to carry out determinations on the tissues of normal rabbits kept under the same conditions as the injected animals. The results recorded in Tables I and II represent, therefore, values for Bayer 205 plus other “amine-precursors”, but the values for normal tissues are sufficiently constant (cf. Table I) to justify the subtraction of “blanks” for each tissue, to give values which represent the Bayer 205 content of the tissues of the injected rabbits (Fig. 1). The source of these amines present in hydrolysed normal tissues has not been investigated, but it is probable that they originate mainly from protein, as they do in the case of normal plasma. The recovery of Bayer 205 added to various normal tissues is not as good as the recovery from plasma, but it is sufficiently satisfactory for the purposes of this investigation. Amounts of Bayer 205 equal to those found in the tissues of the injected rabbits are recovered to the extent of 80–90% with most tissues (kidney, heart, brain etc.), but the recovery with liver tissue is only 55–65%.

In these experiments on “storage” in the tissues, and in many others, Antrypol has been used, and in this work the two terms (Antrypol and Bayer 205) are used synonymously.

The Antrypol content of various organs and tissues following the injection of the drug into rabbits

Three groups of rabbits received intravenous injections of Antrypol solutions at intervals; at each injection 0.03 g. Antrypol/kg. was given. The injections were made at intervals of 3 weeks, and two groups were killed 3 weeks after they had received the last injection. The third group, that receiving three injections, was left for 10 weeks after the last injection, since it was thought advisable to avoid a high plasma-Bayer 205 level which would cause high values

¹ The authors would like to take this opportunity of thanking Dr J. L. D'Silva of the Department of Physiology for his generous assistance with these operations.

to be obtained for tissues rich in blood. The weights of the rabbits ranged from 1.7 to 3.1 kg. The details of the injections can be tabulated as follows:

	No. of rabbits	No. of injections	Intervals between injections days	Interval between last injection and death days
Group A	3	1	—	21
Group B	3	2	21	21
Group C	4	3	21 and 21	70

Two control rabbits for each group were kept under similar conditions with regard to diet etc., and were subsequently treated in the same way as the injected animals. The results of this experiment are given in Tables I and II and in Fig. 1. With the injected rabbits, as with the controls, there is good agreement between two samples of the same organ, although these samples were often taken from different parts of the organ concerned. If the values for the kidney are excluded, the average difference between duplicates of this type is less than 0.04 mg./g. of tissue.

The "true" Antrypol values for the organs of the injected rabbits are plotted in Fig. 1. These figures represent the differences between the averages for each organ of each group of injected rabbits and the corresponding average "blank" values for the control animals (last column, Table I). The results obtained show that there is a small amount of the drug in each of the organs examined, with the possible exception of the brain. Part of the Antrypol present in each tissue can certainly be attributed to the blood and tissue fluid of that organ, but there is usually sufficient to indicate a slight retention of the drug in the tissues in addition to that present in the blood. It is suggested that this retention is due to combination with tissue proteins, but with two exceptions the amount of drug retained in any one organ is very small. The values for the kidney, however, are considerably higher than those for any other organ or that for plasma, and in addition there is distinct variation between the values for different rabbits of the same group (cf. Table II). The spleen also gives higher results than those for

Table I. *Amines produced by acid hydrolysis of tissues of normal rabbits*

Calculated as mg. Bayer 205/g. tissue or/ml. of plasma

	No. 170	No. 177	No. 185	No. 188	No. 297	No. 299	Average
Kidney	0.070 0.031	0.033 0.029	0.048 0.052	0.042 0.043	0.039 0.039	0.041 0.044	0.043
Liver	0.039 0.041	0.042 0.046	0.050 0.050	0.035 0.039	0.032 0.034	0.038 0.036	0.040
Heart	0.033 0.034	0.033 0.033	0.055 0.039	0.037 0.038	0.047 0.047	0.048 0.040	0.040
Muscle*	0.022 —	0.030 —	0.034 0.023	0.024 0.027	0.035 0.032	0.016 0.015	0.026
Lung	0.048 0.050	0.032 0.035	0.035 0.041	0.034 0.036	0.036 —	0.042 0.039	0.039
Brain	0.036 0.028	0.022 0.034	0.029 0.026	0.028 0.027	0.028 —	0.025 0.026	0.028
Adrenals	0.033	0.029	0.012	0.034	0.016	0.015	0.023
Spleen	0.054	0.063	0.061	0.069	0.042	0.070	0.060
Pancreas	0.031	0.016	0.024	0.029	0.030	0.032	0.027
Plasma	0.008	0.008	0.008	0.008	0.008	0.007	0.008

* From the abdominal wall.

Table II. *Tissues of rabbits injected with Antrypol (Antrypol plus other amine-precursors in terms of mg. Bayer 205/g. tissue or/ml. of plasma)*

	Group A (1 injection) Rabbit no.			Group B (2 injections) Rabbit no.			Group C (3 injections) Rabbit no.			
	244	282	283	229	237	241	285	289	301	317
Kidney	0.239 0.210	0.139 —	0.111 0.114	0.111 0.122	0.290 0.310	0.124 0.103	0.104 0.105	0.119 0.116	0.099 0.102	0.116 0.123
Liver	0.059 0.047	0.069 0.068	0.049 0.047	0.064 0.056	0.067 0.063	0.060 0.055	0.038 0.041	0.055 0.056	0.044 0.041	0.057 0.058
Heart	0.062 0.059	0.066 0.068	0.058 0.053	0.061 0.073	0.063 0.064	0.062 0.062	0.054 0.049	0.062 0.064	0.047 0.049	0.057 0.049
Muscle	0.035 —	0.052 —	0.043 0.040	0.061 —	0.058 0.046	0.053 0.047	0.018 0.026	0.030 —	0.014 0.017	0.017 0.024
Lung	0.060 0.065	0.056 0.056	0.053 0.050	0.071 0.071	0.068 0.061	0.072 0.068	0.040 0.044	0.051 0.049	0.050 0.050	0.055 0.055
Brain	0.029 0.029	0.029 0.032	0.026 0.029	0.034 0.032	0.027 0.032	0.027 0.029	0.026 0.026	0.031 0.027	0.027 0.028	0.024 0.025
Adrenals	0.035	0.047	0.047	0.072	0.063	0.054	0.026	0.030	0.029	0.073
Spleen	0.129	0.107	0.089	0.155	0.091	0.114	0.073	0.115	0.123	0.167
Pancreas	0.034	0.043	0.034	0.059	0.045	0.046	0.034	0.041	0.034	0.033
Plasma	0.040	0.037	0.031	0.056	0.048	0.049	0.020	0.020	0.019	0.022

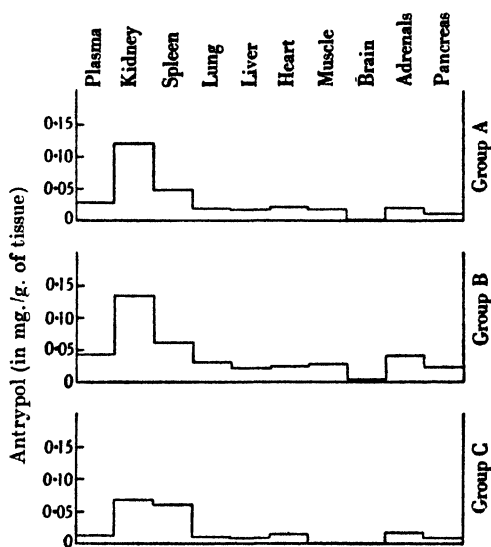


Fig. 1. The Antrypol content of the tissues of rabbits which had received injections of the drug.

plasma, but not as high as those for the kidney. In the case of two of the rabbits, determinations were also carried out with bone marrow and a portion of small intestine; values similar to those for heart, liver, muscle etc. were obtained.

The combination of Bayer 205 with plasma proteins

For this investigation several methods have been used. In some experiments Bayer 205 has been added to serum (rabbit and horse), and in others blood was taken from rabbits which had previously received injections of the drug. The

plasma and serum proteins were then separated by various methods and the amount of Bayer 205 in each fraction was determined. In many of the experiments no attempt was made to find the relative combining capacities of the different proteins for Bayer 205, the main object being to determine whether the drug can combine with plasma proteins. In view of the anticoagulant action of this compound, however, particular attention has been paid to the fibrinogen fraction.

(a) *Bayer 205 added to serum.* Normal horse serum was treated with 0.04 vol. of a strong Bayer 205 solution and kept at room temperature for 1 hr.; the globulins were then separated from portions of this mixture by CO_2 . The results recorded in Table III indicate that a considerable amount of the added drug attaches itself to the globulins. Washing with water saturated with CO_2 , or reprecipitation with CO_2 , removes part only of the "attached" drug, and the observed loss is largely due in the first case to solution of the globulin-drug complex, and in the second to the difficulty in effecting complete reprecipitation of the globulin.

Table III. *Separation of the globulins from serum to which Bayer 205 has been added*

	mg. Bayer 205/100 ml. serum*			
Whole serum (horse)	93	1.7	48	97
Globulin fraction (CO_2)	53	1.2	17	26
Globulin fraction (washed)	46	1.2	17	22
Globulin fraction (reprecipitated)	35	1.2	13	21

Experimental details. A mixture of 1 vol. of serum and 9 vol. of distilled water was cooled in ice-water, and the globulins were precipitated by passing in CO_2 to give maximum precipitation. The mixture was kept in ice-water for 20 min. and centrifuged. The deposit was dissolved in 1 vol. of 0.9% NaCl with the aid of sufficient dilute NaOH to give pH 7.5.

Some of the globulin precipitates were washed with CO_2 -saturated distilled water, and in other cases reprecipitation was effected by dilution and treatment with CO_2 .

* In these experiments and those recorded in Tables IV-VI, blank determinations have been made on the corresponding fractions of normal plasma or serum, and compensation has been made for the small amount of amine-precursors present in some of these controls.

In other experiments the drug was added to horse (and rabbit) serum and the proteins precipitated by alcohol. Precipitation was effected by adding 3 vol. of absolute alcohol, and the precipitates were washed with absolute alcohol (4 vol.) and finally with ether (4 vol.). With greatly varying amounts of Bayer 205 (1-90 mg./100 ml.) the protein precipitates contained almost all (93-95%) of the added drug.

(b) *The plasma of rabbits injected with Bayer 205.* Blood was taken from rabbits which had previously received one or more intravenous injections of Bayer 205, the conditions of the experiment being varied as much as possible to obtain a wide range of plasma-Bayer 205 values.

Precipitation of the plasma proteins by alcohol gave preparations which contained 95-100% of the plasma-Bayer 205. This combined or adsorbed Bayer 205 was not removed to any marked extent by washing with alcohol, and less than one-third was removed when the precipitates were washed with aqueous alcohol (Table IV).

In other experiments various protein fractions (fibrinogen, fibrinogen plus globulin, and globulin) have been separated from Bayer 205-containing blood from injected rabbits. The results recorded in Table V show that an appreciable

Table IV. *Bayer 205 content of alcohol-precipitated proteins of the plasma of rabbits which had received injections of the drug*

Plasma	mg. Bayer 205/100 ml. plasma								
	2.9	1.7	10.3	12.8	7.75	6.8	5.0	4.75	3.75
Precipitated proteins:									
(a)	2.85	1.55	9.7	12.6	7.8	6.6	—	—	—
(b)	—	—	9.1	11.9	—	6.5	4.8	4.55	3.65
(c)	—	—	—	—	—	—	3.45	3.25	2.85

Experimental details. 2 ml. oxalated plasma were treated with 6 ml. of absolute alcohol, mixed well and centrifuged. Some of these precipitates were treated as follows:

(a) Dried *in vacuo* over CaCl_2 .

(b) Washed with 8 ml. of absolute alcohol, then with 8 ml. of ether and dried at room temperature.

(c) Washed successively with 8 ml. of "75% alcohol" (1 vol. of water plus 3 vol. of alcohol), 8 ml. of absolute alcohol and 8 ml. of ether; dried at room temperature.

part, but not the whole, of the drug present in the plasma is attached to the globulin. Of the other plasma proteins the most interesting in this connexion is fibrinogen, since it has been suggested that the anticoagulant action of Bayer 205 is due to the combination of the drug with fibrinogen. It has previously been noted, however, that plasma and serum obtained from the same blood contain the same amount of Bayer 205 [Bournsnel *et al.* 1939], and further determinations covering a much wider range of Bayer 205 levels, and with considerable variation in the number of injections have fully confirmed this observation (cf. Table VI). From these results and from determinations on fibrinogen (fibrin) precipitated by the addition of CaCl_2 to the diluted plasma (Table V), it is concluded that under the conditions of these experiments no significant part of the plasma-Bayer 205 is closely attached to the fibrinogen. The small amount present in the precipitated fibrin may possibly be associated with some component of fibrin other than fibrinogen.

Table V. *Bayer 205 content of separated proteins of the plasmas of injected rabbits*

	mg. Bayer 205/100 ml.							
	2.9	1.7	5.0	3.9	—	—	—	—
Serum	—	—	—	—	—	—	—	—
Plasma	2.9	1.7	—	—	18.2	22.2	15.2	12.2
Fibrinogen (fibrin)	0.3	0.3	—	—	1.0	0.9	0.8	0.3
Fibrinogen plus globulin	1.0	0.75	—	—	2.85	2.6	3.0	1.7
Globulin	—	—	1.5	0.95	—	—	—	—

Experimental details. Fibrinogen (fibrin) was separated by the addition of CaCl_2 to plasma diluted with 0.85% NaCl , and fibrinogen plus globulin by the addition to plasma of 19 vol. of 27.79% $(\text{NH}_4)_2\text{SO}_4$ solution [for full details, cf. Harrison, 1937]. Serum globulin was separated by the addition of 1 vol. of water and 2 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and the precipitate was washed with about 3 vol. of half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution.

Table VI. *Comparison of plasma and serum (of injected rabbits)*

	mg. Bayer 205/100 ml.							
	7.0	9.3	5.4	4.5	28.4	15.0	21.1	15.8
Plasma (oxalated)	7.0	9.3	5.4	4.5	28.4	15.0	21.1	15.8
Plasma (heparinized)	—	9.3	—	4.6	—	15.0	20.3	—
Serum	7.0	—	5.2	—	28.6	—	—	16.0

Experimental details. Samples of 2.5, 5.0 and 2.5 ml. of blood were taken from each rabbit. The first and third samples were oxalated and mixed; the second sample was collected in a heparinized tube (containing 0.05 ml. of 1% heparin solution) or in a clean tube (for serum separation).

DISCUSSION

Little information is available as to the fate of Bayer 205 in the animal body, and although it is known that a considerable amount of the injected drug remains in the body, the cause of this retention is still rather obscure. Sei [1923] has shown that a trypanocidal action is exerted by extracts of certain organs of animals which have received large doses of Bayer 205, and chemical tests have been used to detect the drug in the tissues of the injected animals [Zeiss & Utkina-Ljubowzewa, 1930; Demidowa, 1931]. These chemical tests were made, however, by a method which does not appear to be satisfactory for the determination of Bayer 205 [cf. criticism by Lang, 1931; Dangerfield *et al.* 1938], and, furthermore, in practically all these earlier investigations the animals were injected with large doses of the drug.

The method of determination of Bayer 205 evolved in previous papers [Dangerfield *et al.* 1938; Bournsnel *et al.* 1939] can be utilized for determinations on tissues. The recovery of added Bayer 205 is not as satisfactory as it is in the case of plasma, but is sufficiently high (80–90 % with most tissues) for practical purposes. The method will detect very small amounts of Bayer 205 and it has been possible, therefore, to limit the amount of injected drug to a normal prophylactic dose. At each injection the rabbits used in this investigation received 0.03 g. of drug/kg., equivalent to a dose of about 2 g. for an average man. One disadvantage of this or any similar chemical determinations is that it does not differentiate between Bayer 205 and possible modifications of the drug, but there is no evidence as yet that degradation of Bayer 205 occurs in the animal body. It is hoped in a later publication to deal with this question of the possible "metabolism" of this drug.

The determinations of the Antrypol (or Bayer 205) content of the tissues of rabbits which had previously received one, two or three injections of the drug show that there is no preferential storage of the drug in the liver or any other organ examined, with the possible exception of the kidney. The amount of drug in the liver, heart, muscle, lung, adrenals and pancreas is very small, and the average value for each of these tissues is usually 0.5–0.9 times that present in an equal weight of blood (cf. Fig. 1). The drug present in the blood and tissue fluid can undoubtedly account for an appreciable part, but not all, of the total drug present in each of these organs. It seems probable, therefore, that a very small amount of the drug is held in each tissue, partly in combination with the proteins of blood and tissue fluid, and possibly associated with the tissue proteins; it is significant in this respect that brain tissue which contains very little protein contains little or no Antrypol. The Antrypol content of the liver and other similar tissues is, however, very small, and represents only a fraction of the injected drug. Three weeks after the injection of 30 mg. of Antrypol/kg. of body weight, the liver contained about 0.8 mg. and the heart 0.1 mg. of the drug (group A). The corresponding figures, 3 weeks after a second injection of the same amount (group B), are 1.2 and 0.1 mg. respectively, and for the third group, where three injections were given and the rabbits were killed 10 weeks after the third injection, the values were 0.6 and 0.07 mg. respectively. The corresponding values for the whole of the blood, assuming a blood volume of approximately one-twelfth of the body weight, are 5.5, 8.3 and 2.4 mg. for groups A, B and C respectively. As was mentioned above, these experiments were carried out to determine whether there is appreciable storage of Bayer 205 in any particular organ, and it was not intended that they should serve as balance experiments to determine exactly how much of the drug is retained in various parts of the body.

Experiments of an entirely different nature are being carried out with the latter object in view, but from the observations made in the present paper it can be concluded (a) that the drug is fairly widely distributed throughout the body, and (b) that for several weeks after the injection of the drug a significant part of the injected material is still present in the animal. It is hoped that subsequent investigations on the urinary excretion of the drug by man, and other animals, will indicate how much of the injected drug remains in the body after a period of several weeks.

Of all the organs examined, the kidney occupies a special position. The values obtained suggest that significant amounts of the injected drug often accumulate in this organ, particularly in certain animals; thus one rabbit (no. 244), which received 82 mg. of Antrypol, had 2.2 mg. of the drug in its kidneys 3 weeks later, and another rabbit (no. 237) which received two injections each of 90 mg., had 3.8 mg. of the drug in its kidneys 3 weeks after the second injection. The presence of appreciable amounts of the drug in the kidney is of special interest in view of the fairly frequent occurrence of albuminuria in patients treated with the drug. There is also evidence that the injection of Bayer 205 into healthy mice causes extensive degeneration in the epithelium of the convoluted and other secreting tubules of the cortex of the kidneys [Duncan & Manson-Bahr, 1923-4].

The amount of drug in the spleens of the injected animals is not as high as that in the kidneys, but it is significantly higher than the values for the other organs examined. Part of this difference can be attributed to the high blood content of the spleen, but it is possible that some additional factor is concerned. There is much recent work which suggests that the spleen, and the reticulo-endothelial system in general, play an important role in the chemotherapeutic action of certain trypanocidal drugs, and it is claimed that with rats and mice, splenectomy destroys the sterilizing action of various trypanocidal drugs [for the literature see Findlay, 1939, p. 231]. According to Kritschewski [1928] combination of the drug with agar gives effective results in these splenectomized animals, presumably by the formation of a depot from which slow absorption occurs. There is also evidence that Bayer 205 may exert its action by an opsonin-like effect, producing a change in the trypanosomes which renders them more readily phagocyted [Reiner & Köveskuty, 1927; Jancsó & Jancsó, 1934; 1935; Hawking, 1939]. According to this view the reticulo-endothelial system would play an active part in the trypanocidal effect but it would not necessarily suggest that Bayer 205 combines with the reticulo-endothelial system. Although they offer no evidence as to the role of the spleen in the chemotherapeutic action of Bayer 205, the experiments described in the present paper indicate that there is no extensive storage of the drug in the spleen.

From the above results it is concluded that the long retention of Bayer 205 in the animal body following intravenous injection of the drug is not due to storage in any particular organ, but to a more general retention in the blood and tissues. One possible explanation for this retention is that the drug combines with the plasma- and tissue-proteins, and for many reasons this possibility has been under consideration throughout this work. It is of interest to note that in some of the earliest investigations on Bayer 205 it was suggested that the drug is bound up with the serum proteins, and a considerable amount of indirect evidence in support of this view has been obtained by many workers in investigations on the trypanocidal, anti-complementary and anti-coagulant action of the drug. In certain concentrations, Bayer 205 prevents the heat-coagulation of blood proteins [Collier, 1925] and it also protects these, and other proteins, against

precipitation by tannin, mercuric chloride and certain other reagents [Jirovec & Kocian, 1930; Kocian, 1936]. Klopstock [1932] has investigated the effect of Bayer 205 and of heparin on immune reactions and concludes that both substances shift the isoelectric point of certain of the immune substances to the acid side, and that in higher concentrations they react with specific groups in the protein molecule. There is also evidence that Bayer 205 specifically inactivates certain enzymes. Quastel [1931], for example, found that it is toxic to fumarase but not to urease, and he suggested that there is some structure in common between the fumarase enzyme, cotton fibre and the trypanosome which makes for specific combination or adsorption with certain naphthylaminedisulphonic acid derivatives. Fürth *et al.* [1932] also report that Bayer 205 is not a general enzyme poison but that it accelerates post-mortem production of acid in muscle and liver, and the bacterial production of lactic acid in milk.

In the experiments reported in this paper, evidence is produced that Bayer 205 is closely associated with plasma proteins. It has been found that the proteins precipitated from normal plasma or serum after the addition of the drug, or from the plasma or serum of injected rabbits, contain appreciable amounts of Bayer 205. For obvious reasons the methods of precipitation were varied as widely as possible, and it is believed that there can be no possibility that in every case the drug was carried down by "simple" adsorption. If, however, it is suggested that adsorption accounts in every case for the drug present in the precipitates it would still indicate a fairly strong chemical affinity between the protein and the drug. The proteins precipitated by the addition of 3 vol. of alcohol contain practically all the Bayer 205 present in plasma or serum, and the drug is only partially removed when the precipitate is washed with "75 %" alcohol (in which Bayer 205 is quite soluble). The globulin precipitates obtained with ammonium sulphate also retained a high proportion of the drug when they were washed with ammonium sulphate solutions, and solution and reprecipitation of the globulin fractions yielded precipitates with a high Bayer 205 content. The conclusion is reached, therefore, that the drug combines with, or is specifically adsorbed by, some of the plasma proteins.

No effort has so far been made to determine the distribution of the drug between the different plasma proteins, but investigations along these lines are now being made. The results recorded in this paper, and others which will be reported later, indicate that there is no special affinity between fibrinogen and Bayer 205 under the conditions of our experiments. Fibrinogen, precipitated from Bayer 205-containing plasma as fibrinogen or as fibrin, contains a small amount only of the drug, and it is possible that part of this small quantity is associated with non-fibrinogen constituents of the precipitate. Perhaps more conclusive, however, is the fact that plasma and serum derived from the same Bayer 205-containing blood contain exactly the same amount of the drug; from this it can be concluded that no appreciable amount of the drug is attached to the fibrinogen, unless it is assumed that any Bayer 205-fibrinogen complex is broken down when fibrinogen is converted into fibrin to form a clot. Since the latter possibility appears rather unlikely, these results suggest that the anti-coagulant action of Bayer 205 is not due simply to its combination with fibrinogen. Further studies are being made to determine which constituents of the blood-clotting system combine with this drug, and it is hoped that further information will also be obtained with regard to the mechanism of the anti-complementary action of the drug.

SUMMARY

1. Investigations have been made to determine the cause of the long retention of Bayer 205 (or Antrypol) in the animal body after injection of the drug. In particular, the possibilities of (a) storage in certain organs, and (b) combination with plasma- and tissue-proteins have been studied.

2. There is no marked storage of the drug in the liver, heart, muscle, lung, brain, adrenals or pancreas of rabbits which have received one, two or three injections of Antrypol. Each of these organs, except the brain, retains a small but measurable amount of the drug, possibly in combination with the tissue proteins.

3. The kidneys of these injected rabbits contain considerably more Antrypol than do the other tissues examined, and the amount present (in terms of mg. drug/g. kidney) varies appreciably from one animal to another. This retention of the drug in the kidney is of special significance in view of the fairly frequent occurrence of albuminuria following the injection of the drug into man.

4. The spleen contains a little more of the drug than do the other organs examined, but considerably less than the kidney. Slight retention in the spleen might possibly be due to association of the drug with the reticulo-endothelial system.

5. Various protein fractions have been separated from the serum and plasma of injected rabbits, and from normal serum to which Bayer 205 has been added. Evidence has been obtained that plasma globulin and probably other proteins can combine (by adsorption or otherwise) with this drug.

6. The conclusion is reached that the long retention of Bayer 205 in the animal body is due to the combination of the drug with plasma and tissue proteins.

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CXLVII. THE COMPOSITION OF FORAGE CROPS

III. COCKSFOOT. CHANGES IN THE HERBAGE DURING GROWTH, WITH AND WITHOUT ADDITIONS OF NITROGENOUS FERTILIZER

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THE digestibility of forage crops varies widely with the age and composition of the tissues. A study of the developmental changes in the composition of rye grass [Norman, 1936; Norman & Richardson, 1937], using some newer analytical procedures, showed that apart from the diminution of protein content and the increase in lignin content, well recognized as accompanying the onset of maturity, important changes occurred in the carbohydrates. A water-soluble fructosan was found to be present in considerable amount in immature grass, apparently acting as a temporary reserve, later to be utilized for the production of insoluble structural constituents, predominantly cellulose. Such a change would substantially affect the nutritive value and digestibility of the herbage, but is not clearly evident from the results of the conventional analyses applied to feeding stuffs, as has been pointed out elsewhere [Norman, 1937, 1]. The purpose of the investigations reported here was to follow analytically the changes in composition during growth of another grass, cocksfoot (*Dactylis glomerata*; known in the U.S.A. as orchard grass) which is generally regarded as being coarser and less palatable than rye grass, and to determine if the latter is peculiar in its high content of fructosan or if this polysaccharide has an equally important role in another member of the Gramineae. Some evidence having been obtained in the case of both rye grass and barley that applications of N cause a reduction in the percentage content of fructosan, direct comparisons were made of grass taken from untreated and N-fertilized plots. Analyses were also carried out on the second growth of this grass by re-cutting all plots 4 weeks later.

Plan of experiment and source of samples

The experimental area was located on the north headland of Long Hoos field (section IV), Rothamsted, and consisted of a pure stand of cocksfoot in its second season. 160 small plots each $1\frac{1}{2}$ yd. square were laid out in two adjacent blocks each containing 80 plots. As before an area of 1 sq. yd. was cut from the plot at the time of sampling, and, because of the margin remaining, edge effects could be ignored. In view of the variation in yield obtained in the rye grass experiment in which only 6 plots were cut to form the composite sample, the number was increased to 8, which were chosen at random. The bulked samples were treated as in the previous season.

Sampling was commenced on 10 May 1937, when 8 plots were cut on each block. One block then received an application of sulphate of ammonia at

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the rate of 2 cwt. per acre. Each week until 28 June 8 plots were cut on each block. Supplementary applications of sulphate of ammonia at half the rate above were applied to the N block on 24 May and 7 June. The rainfall was well distributed and the grass made excellent growth. The month of May in this year was distinctly wetter than the average, rain falling on 15 days. All the plots were cut a second time 4 weeks after the first cut and bulked in the same combinations as before. In the following Tables sample numbers with the suffix N refer to grass taken from plots in the block receiving applications of sulphate of ammonia, and with the suffix O, from the untreated block. It is to be noted that all changes due to the application of N occurred after 10 May, since up to that time the treatment of the blocks had been identical.

Yield and composition of cocksfoot

The rate of growth of the grass is shown in Table I, the yield being expressed as dry weight per sq. yd. A substantial increase in dry weight was apparent in 3 weeks as a result of the application of N, and taken at the point of maximum yield the N plots outyielded the untreated plots by almost one-third. With additions of N the final yield of mature grass exceeded 2 tons per acre on the dry basis.

Table I. *Yield of cocksfoot per sq. yd. (first cut)*

Date of cutting	Sample	Oven-dry weight g.	Sample	Oven-dry weight g.
10 May	1-O	90	—*	—
18 "	2-O	130	2-N	124
24 "	3-O	166	3-N†	186
31 "	4-O	196	4-N	277
7 June	5-O	257	5-N†	377
14 "	6-O	284	6-N	413
21 "	7-O	335	7-N	456
28 "	8-O	314	8-N	460

* Ammonium sulphate applied at the rate of 2 cwt./acre.

† Ammonium sulphate applied at the rate of 1 cwt./acre.

Table II. *Some non-structural constituents of cocksfoot (first cut). All analyses expressed as % on oven-dried material*

Sample	Ash	Crude protein	Cold water-soluble	Protein in aqueous extract	Fructose before hydrolysis	Fructose after hydrolysis	Alcohol-benzene extract
1-O	10.8	12.3	33.9	4.4	2.7	11.3	18.7
2-O	9.7	11.0	32.1	4.1	3.6	11.1	16.9
2-N	10.1	15.4	31.8	6.8	3.5	9.4	18.3
3-O	9.3	9.9	28.3	3.3	2.8	8.7	13.0
3-N	9.7	12.1	25.0	3.8	2.6	5.8	12.6
4-O	9.8	7.5	24.2	2.3	3.1	8.3	13.0
4-N	10.2	12.3	23.5	3.0	3.4	4.8	16.0
5-O	8.5	7.0	23.6	2.3	3.6	7.5	14.2
5-N	8.1	10.3	22.8	3.4	3.4	5.7	13.4
6-O	8.0	5.3	20.7	2.1	2.6	6.9	12.3
6-N	7.9	10.6	21.3	3.1	2.7	5.0	14.4
7-O	7.0	5.4	22.3	2.3	2.1	7.8	12.1
7-N	8.8	9.1	24.0	3.1	2.9	7.3	13.0
8-O	7.8	5.4	23.6	2.2	1.9	9.7	11.0
8-N	8.0	7.9	24.0	2.5	2.9	8.2	13.8
S.E. mean ±	0.09	0.15	0.21	0.05	0.04	0.06	—

Considering first some non-structural constituents (Table II), the N content of the herbage from the untreated plots diminished steadily, as is usually observed. The application of N was rapidly reflected in an increased protein content, even in as short a period as 1 week, and the protein content of the herbage receiving N was in all cases substantially higher. On an area basis the maximum yield of protein was obtained in sample 6-N, equivalent to 467 lb. per acre. In the untreated grass the peak was only 193 lb. in sample 7-O.

The water-soluble material in the grass was distinctly less in amount than in rye grass and was not much affected by the addition of N. It might have been expected that an increased N content would be accompanied by an increase in soluble constituents, but no indication of this was obtained. Indeed, in considering the fructosan, which in rye grass forms the major water-soluble constituent, the reverse appears to be the case, the addition of N to the herbage in every case having depressed the fructosan content. Unlike the rye grass too, the highest content was not found at the time of rapid emergence of the head, but about 3 weeks earlier in the first sample collected, when it amounted to only 11.3% as against a peak of over 30% in rye grass. The influence of N in reducing the accumulation of fructosan in barley has been described by Archbold [1938], who found that the fructosan content was highest when the N level was low. If fructosans are of secondary origin and arise only when the demand for soluble sugars in other parts of the plant is satisfied, as this author suggests, the addition of N might be expected to have the result observed by stimulating more extensive growth. This, however, is probably too simple a view. Although the peak content of fructosan in this grass was far lower than in rye grass, this constituent did not become so depleted on maturity. The fully mature samples still contained 8-9%, and indeed there were indications of a slight rise in the final stages. No importance is attached to the amounts of free fructose found in these samples, since this is affected by the drying procedure and is considerably higher than that found by extraction of fresh material. Indications were obtained of the presence of yet another polysaccharide in the aqueous extract, as was the case also in rye grass. A careful study of the water-soluble carbohydrates of grasses would be desirable, since there is obviously some constituent unaccounted for.

Extraction with hot 1 : 1 alcohol-benzene was carried out primarily as a pre-treatment in the determination of lignin. Even in the mature samples the amount removed by the reagent was appreciable and consisted, of course, of a mixture of several constituents.

Analyses for the structural constituents are given in Table III. The cellulose content of this grass throughout its whole development was considerably higher than that of rye grass and this is expressed in the general belief that cocksfoot is a "coarse" grass of high fibre content. No large differences as a result of the N treatment were to be seen, though in most cases samples from plots so treated had a slightly lower cellulose content. Cellulose was at all times easily the largest single constituent. As the cellulose content increased the ratio of pentose to hexose groups in the newly formed cellulose also changed so that the amount of xylan in the cellulose rose from 25.7% in the young grass to nearly 30% in samples 5 and 6, thereafter declining a little. Similar changes in the nature of the cellulosic framework of the plant have been observed in both rye grass and barley, but at present no explanation of this change can be offered.

The direct determination of encrusting polyuronide hemicelluloses has not yet been achieved, and accordingly the furfuraldehyde yield from pentose and uronic groups not associated with the cellulose must still be used as a measure of

Table III. *Some structural constituents of cocksfoot (first cut). All analyses expressed as % on oven-dried material*

Sample	Cellulose	Furfur-aldehyde from cellulose	Xylan in cellulose	Total furfur-aldehyde	Furfur-aldehyde from polyuronides	Lignin	Nitrogen in lignin
1-O	38.5	6.3	9.9	10.2	4.0	7.9	0.5
2-O	42.4	7.2	11.2	11.3	4.1	7.1	0.4
2-N	41.3	7.2	11.1	10.8	3.7	7.0	0.5
3-O	44.4	7.8	12.1	12.4	4.6	8.6	0.5
3-N	46.0	8.3	12.9	12.7	4.4	8.7	0.6
4-O	46.8	8.7	13.4	13.8	5.2	10.2	0.4
4-N	44.7	8.0	12.4	13.1	5.1	11.1	0.5
5-O	45.8	8.7	13.5	13.5	5.8	11.1	0.4
5-N	45.8	8.7	13.5	14.0	5.3	12.0	0.5
6-O	48.9	9.1	14.1	14.0	4.9	11.1	0.3
6-N	46.2	8.9	13.8	13.4	4.4	12.4	0.6
7-O	48.6	9.0	13.9	14.1	5.1	11.9	0.3
7-N	45.6	8.5	12.3	13.1	4.6	12.4	0.5
8-O	46.4	8.6	13.3	13.6	5.0	12.1	0.2
8-N	44.3	7.9	12.3	12.6	4.8	11.8	0.4
S.E. of mean \pm	0.13	0.04	0.06	0.10	—	0.08	—

this constituent. The approach of maturity was not accompanied by any substantial increase in the hemicelluloses. The amount present in the herbage which had received applications of N was always slightly lower than in that from the untreated plots.

It has been shown in earlier studies that the deposition of lignin occurs slowly and steadily throughout the development of the plant. This was obviously the case in cocksfoot, though the amount, 7.9%, found in the youngest samples was surprisingly high. Lignin is slightly over-estimated by the procedure used because of a disturbance caused by the presence of protein [Norman, 1937, 2], and although the herbage treated with N appeared to have a slightly higher lignin content, it is doubtful whether the differences are significant, since in every case more N was retained by the lignin residue.

Taking the structural constituents together, there is less difference between the immature and mature cocksfoot samples than was found between corresponding rye grass samples. That is to say, cellulose and lignin together increased only from 46 to 58% in the former as compared with an increase of 29 to 47% in the latter in an equal time interval. An interesting relationship between fructosan and structural constituents, particularly cellulose, appeared to hold in rye grass, the former being apparently utilized and transformed into the latter after the plant had ceased increasing in dry weight. There is no indication of a similar relationship in cocksfoot (Fig. 1), either with or without applications of N. When expressed on the basis of g. per sq. yd., both cellulose and fructosan continued to rise to maturity, the latter rather more rapidly in samples 7 and 8 when cellulose deposition was slackening. Although the percentage of fructosan in cocksfoot was distinctly lower than in rye grass, the maximum amount on an area basis was of the same order because of the heavier growth of the grass. Sample 8-N contained fructosan amounting to 402 lb. per acre, and sample 8-O, to 324 lb. per acre. If on nutritional grounds it was found that the fructosan was a particularly desirable constituent in herbage, then applications of N

might have to be considered carefully, inasmuch as in cocksfoot, though the percentage content is diminished, the yield on an area basis is increased after a certain stage (Fig. 2).

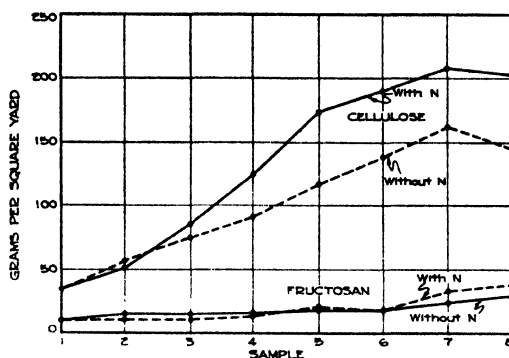


Fig. 1. Changes in cellulose and fructosan in cocksfoot expressed on an area basis.

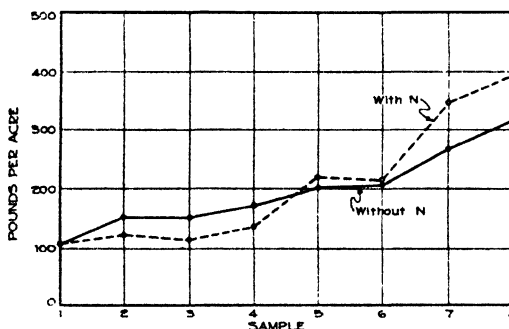


Fig. 2. Yield of fructosan in cocksfoot with and without nitrogen applications.

Yield and composition of second growth cocksfoot

Samples from the re-cut areas must be compared on a different basis from first growth samples, inasmuch as they do not represent material of increasing maturity, but instead they are all of equal age, the 4-week period of growth moving progressively later. Any differences that may be found must then be due to the fact that the root systems from which the second growth developed were at different stages of maturity, and that soil and climatic conditions were variable. Since the plots cut were distributed at random within the blocks it was not feasible to apply late dressings of N at equivalent times. As described earlier, blanket applications were made to the whole block on 24 May and 7 June. The first would only directly affect the second growth on the plots pooled to form samples 1-3, and the latter that on the plots pooled to form samples 2-5. The later samples were therefore completely dependent on residual soil N.

The yields of second growth grass on the same groups of plots as originally sampled are given in Table IV. Both with and without added N the yields decreased as the season progressed. A substantial N effect was apparent, the highest yield, as might be expected, being obtained on the plots initially cut early and supplied with N. All the samples had a high ash content. The protein content of the herbage from the untreated plots (Table V) changed little as the

Table IV. *Yield of cocksfoot per sq. yd. (4 weeks' second growth)*

Date of cutting	Sample	Oven-dry weight g.	Sample	Oven-dry weight g.
7 June	1-O-1	55.1	1-N-1	137.4
14 "	2-O-1	48.6	2-N-1	119.0
21 "	3-O-1	41.0	3-N-1	122.0
28 "	4-O-1	34.8	4-N-1	106.0
5 July	5-O-1	21.3	5-N-1	67.8
13 "	6-O-1	20.2	6-N-1	61.1
20 "	7-O-1	22.8	7-N-1	44.7
27 "	8-O-1	11.9	8-N-1	24.4

Table V. *Some non-structural constituents of cocksfoot (4 weeks' second growth). All analyses expressed as % on oven-dried material*

Sample	Ash	Crude protein	Cold water-soluble	Protein in aqueous extract	Fructose before hydrolysis	Fructose after hydrolysis	Alcohol-benzene extract
1-O-1	12.9	12.0	27.4	2.8	1.9	6.7	14.1
1-N-1	12.0	19.3	27.5	4.8	1.9	3.8	16.3
2-O-1	13.1	12.4	26.5	3.1	1.4	3.5	15.8
2-N-1	12.4	17.9	25.0	5.0	1.9	2.8	12.9
3-O-1	13.0	12.2	28.8	3.4	1.6	6.1	14.2
3-N-1	12.9	14.3	26.9	3.9	2.3	5.0	15.7
4-O-1	13.4	11.7	28.6	2.3	1.6	6.7	11.9
4-N-1	12.6	14.1	27.9	3.6	1.8	6.0	11.8
5-O-1	13.7	12.0	26.6	2.8	1.1	5.1	13.1
5-N-1	13.4	14.0	27.2	3.7	1.7	5.4	12.5
6-O-1	13.7	12.2	24.6	2.9	1.1	5.6	13.1
6-N-1	12.1	13.6	27.4	3.5	1.8	5.7	11.4
7-O-1	13.0	10.9	23.5	3.0	0.8	3.5	14.4
7-N-1	12.1	12.3	23.7	3.1	1.2	4.4	11.4
8-O-1	12.4	10.1	21.6	1.8	0.9	2.6	13.2
8-N-1	13.0	13.5	24.2	2.9	1.1	3.7	12.9
S.E. of mean \pm	0.09	0.15	0.21	0.05	0.04	0.06	--

Table VI. *Some structural constituents of cocksfoot (4 weeks' second growth). All analyses expressed as % on oven-dried material*

Sample	Cellulose	Furfuraldehyde from cellulose	Xylan in cellulose	Total furfuraldehyde	Furfuraldehyde from polyuronides	Lignin	Nitrogen in lignin
1-O-1	40.1	7.0	10.8	10.6	3.6	8.6	0.5
1-N-1	40.8	6.9	10.7	10.2	3.2	9.0	0.6
2-O-1	43.5	7.7	11.9	11.1	3.6	8.8	0.5
2-N-1	41.0	6.9	10.8	11.0	4.1	10.0	0.7
3-O-1	42.1	6.8	11.3	10.8	3.6	8.2	0.4
3-N-1	40.8	6.9	10.5	10.4	3.6	10.0	0.5
4-O-1	40.5	7.1	10.6	10.2	3.4	8.9	0.6
4-N-1	39.8	7.1	11.0	9.9	2.8	9.1	0.5
5-O-1	42.7	6.7	11.0	10.4	3.2	8.3	0.4
5-N-1	40.8	7.9	10.4	10.2	3.6	8.4	0.5
6-O-1	46.8	7.6	12.3	10.8	2.8	8.5	0.4
6-N-1	44.1	7.8	11.8	10.8	3.2	9.9	0.5
7-O-1	44.8	7.7	12.1	11.8	4.0	9.5	0.4
7-N-1	44.4	7.7	12.0	10.3	2.6	9.1	0.5
8-O-1	44.2	7.7	11.9	10.8	3.1	9.9	0.4
8-N-1	43.8	7.1	11.1	10.6	3.5	9.8	0.5
S.E. of mean \pm	0.13	0.04	0.06	0.10	—	0.08	--

season progressed, whereas that from the plots treated with N, at first over 19 %, decreased steadily. On an area basis the protein differences were very great, sample 1-O-1 containing 70 lb. per acre as compared with 282 lb. per acre in sample 1-N-1. No significant differences were found in the amount of total water-soluble material, though the fructosan content was lower than in young first growth herbage. This is in accord with similar observations on rye grass. In some, but not all, cases the effect of the application of N was again to reduce the fructosan content. The structural constituents were not higher in the second growth than in first growth herbage of approximately equal N content. Material from the plots treated with N had uniformly slightly lower cellulose contents than that from the untreated plots, but there were no consistent or significant differences between the two with respect to lignin or polyuronide hemicellulose contents. The amount of the latter present, as judged by furfuraldehyde yield, seemed unusually low, but this may be misleading in view of the observation that the hemicelluloses of immature tissues may have a lower pentose content than those of more lignified tissues.

In general, it cannot be said of cocksfoot that the composition of second growth herbage is as different from the first growth as seemed to be the case in rye grass, and a lower level of fructosan appears to be the most significant difference.

Recovery of nitrogen in the herbage

Apart from the questions of composition it is interesting to notice the recovery of N in the herbage. Because the applications were made on three occasions at intervals of 2 weeks, the uptake was variable. The results in Table VII were arrived at by determining the differences between the sums of the N contents of the first and second growths in the treated and untreated plots. A total of approximately 95 lb. of N per acre was added in the form of ammonium sulphate and the maximum recovery amounted to about 62 %, the highest figures occurring in samples 4 and 6, which were each cut initially 1 week after an application of N.

Table VII. *Recovery of N in the herbage (first and second growth combined)*

Samples	lb. of added N per acre absorbed
2	35
3	31
4	51
5	47
6	59
7	45
8	37

SUMMARY

1. The changes in composition of cocksfoot cut at weekly intervals up to maturity, with and without applications of ammonium sulphate, have been determined.

2. A water-soluble fructosan was found in this grass, the amount being depressed by applications of N. The maximum content was far lower than in rye grass and occurred in the youngest herbage collected. The most mature samples still contained an appreciable amount of this constituent.

3. Throughout development, the herbage was relatively high in structural constituents, the proportions being little affected by the treatments with N.

There was no apparent relationship between the disappearance of fructosan and the formation of structural constituents, as was the case with rye grass.

4. The composition of second growth herbage of equal age from the same plots was determined. The fructosan content of this material was lower than that of immature first growth but there was little difference in the structural constituents.

The assistance of Miss Joan Hellyer in the sampling and analytical work involved is acknowledged.

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CXLVIII. THE EFFECT OF A PHOSPHORUS DEFICIENCY ON THE PROTEIN AND MINERAL METABOLISM OF SHEEP

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MUCH work has been done on various aspects of the effects of aphosphorosis on both ruminants and other types of mammals, but no complete study of the effect on both protein and mineral metabolism has yet been recorded. The effect of a P-deficient diet on the general health, blood chemistry, appetite and mineral metabolism has been admirably reviewed by Theiler & Green [1931] and Schmidt & Greenberg [1935] and needs no further discussion. On the other hand, little attention has been paid to the protein metabolism. In a study on sheep Woodman & Evans [1930] found that the digestibility of the ration was unchanged by either Ca or P deficiency, similar results being recorded by Forbes [1937] on rats and Kleiber *et al.* [1936] on steers. A diminished utilization of food energy has, however, been recorded [Eckles *et al.* 1926; 1932; Theiler *et al.* 1924; Theiler, 1933, 1, 2; Kleiber *et al.* 1936], cows on P-deficient rations requiring 20% more digestible nutrients than are allowed in the feeding standards to maintain a constant live weight [Eckles & Gullickson, 1927].

The present study was undertaken to find if any disturbance of the protein metabolism followed the ingestion of a P-deficient diet.

Experimental

Four sheep were used, the animals being about 14 months old. The diets fed are shown in Table I. During periods 1 and 4 the rations fed were low in P, with a Ca : P (CaO : P₂O₅) ratio of about 5 : 1. In period 2 the ration was supplemented with Na₂HPO₄, giving a Ca : P ratio of approximately 1 : 1. In period 3 both P and Ca additions were made, the ratio again being about 5 : 1, although both the Ca and P intakes were far in excess of the actual requirements. Periods 1, 2 and 4 were of 6 weeks each, i.e. 2 weeks on transition from the previous diet and 4 weeks on experiment. Period 3 lasted only 2 weeks, analyses being carried out for the whole period.

Table I. *Quantities of food consumed per day, g.*

Food	Periods			
	1*	2	3	4
Beet pulp	400	500	500	350
Starch	200	250	250	250
Blood meal	50	50	50	50
Straw	150	200	200	100
Na ₂ HPO ₄	—	10	10	—
CaCO ₃	—	—	45	—
N intake	13.28	15.05	15.05	12.34
CaO intake	5.39	6.00	32.01	4.51
P ₂ O ₅ intake	1.11	6.43	6.43	0.79

* Sheep no. 2 received more food in periods 1 and 4.

Analyses of urine and faeces were carried out every 2 days, the methods being those used in previous experiments [Morris & Ray, 1939].

Results

Food intake. The quantity of food supplied per day was based on preliminary periods in which an excess was offered and accurate records kept of the actual intake. It will be seen from Table I that the food intake varied with the P content of the diet, the low P diets giving a lower food intake. This fact has been recorded by other investigators [Du Toit *et al.* 1931; Huffman *et al.* 1933; Martin & Peirce, 1934] and may possibly be the result of a diminished secretion of digestive juices with a decreased P intake. There was also a lowered state of general health of the sheep during the P-deficient periods, the sheep being ill-at-ease for the first 3 weeks and growing more lethargic till the end of the 6 weeks period. In period 3, that of the high Ca-high P diet, the animals were definitely "off colour" but the period was too short (2 weeks) for any change in the food intake to be recorded. Similar results with a high Ca diet have been recorded by Bethke [1932], Bethke *et al.* [1933] and Cox & Imboden [1936].

Weight changes. The effect of the diets on the gain in live weight is shown in Table II. In period 2, with the P supplement, the gains are slightly greater

Table II. *Live weight changes, kg.*

Sheep	Periods			
	1	2	3	4
1	+2.25	+2.00	+1.25	-2.50
2	+4.50	+1.35	+2.10	-4.20
3	+1.95	+4.05	-1.55	-2.85
4	+0.50	+3.00	-1.90	-4.85
Mean	+2.30	+2.60	+1.70	-3.60

than in period 1, though the differences are not statistically significant, while in the final low P period a marked loss in weight is found. In period 3 the gains are distinctly lower than in period 2. The present results confirm those of other investigators [Du Toit *et al.* 1931; Godden & Ray, 1937].

Protein digestibility. Table III shows the apparent and true digestibilities of the ingested protein. Only in period 4 is there any alteration in the apparent

Table III. *Digestibility of food nitrogen*

Period	Sheep	Faecal N g.	Intake of N g.	% apparent digestibility	Metabolic faecal N, g.	% true digestibility
1 (low P)	1	6.48	26.56	75.6	6.48	100
	2	7.91	28.16	71.8	7.30	97.8
	3	6.31	23.80	73.4	5.70	97.4
	4	6.63	26.56	75.0	6.08	97.9
2 (P supplement)	1	8.47	30.10	71.8	9.10	>100
	2	8.19	30.10	72.8	9.10	>100
	3	10.85	30.10	63.9	9.10	90.1
	4	8.18	30.10	72.8	9.10	>100
3 (P + Ca supplement)	1	8.90	30.10	70.4	9.10	>100
	2	7.05	30.10	76.6	9.10	>100
	3	8.05	30.10	73.2	9.10	>100
	4	7.51	30.10	75.0	9.10	>100
4 (low P)	1	7.39	24.68	70.0	6.08	94.7
	2	7.86	26.24	70.0	6.48	94.7
	3	7.71	24.68	68.8	6.08	93.4
	4	8.74	24.68	64.6	6.08	89.2

digestibility of the protein, a slight lowering being recorded. When the metabolic faecal N is taken into account, period 4 shows a definite lowering of the true digestibility while period 1 also shows a trend in this direction. It may be noted that in periods 2 and 3, when adequate amounts of P and Ca were fed, the metabolic faecal N calculated from the dry matter intake, using the results of Hutchinson & Morris [1936, 1, 2], is greater than the actual amount of N excreted. Mitchell [1934] claims that in such cases the metabolic faecal N figure must be taken and that the error lies in the analytical figure obtained for the actual N excreted. In the present experiment the actual faecal N excreted is lower than the calculated metabolic N so consistently during periods 2 and 3 that some other explanation must be sought. It is possible that, with a completely adequate diet, the apparent biological value of the protein fed may alter the metabolic faecal N, the higher the biological value the lower being the N excretion. In the present instance blood meal was being fed as the chief source of protein and this has been shown to have a high biological value for ruminants [Morris & Wright, 1933, 1, 2; Morris *et al.* 1936].

From the results it is doubtful if there is any significant difference in the apparent digestibility of the protein ingested. There is, however, no doubt that a deficiency of P in the diet causes a significant lowering of the true digestibility of the protein.

Urinary partition of nitrogen

(i) *Total N.* A P-deficient diet has a marked effect on the total urinary N output (Table IV). In periods 1 and 4, although the N intake is lower than in periods 2 and 3, the urinary output is, on the whole, higher, showing a decreased retention of absorbed N (see also Table VII). In period 3 the N excretion is significantly higher than in period 2, although the N intake is the same. This indicates that the Ca : P ratio, together with the total P intake, has a definite influence on the protein metabolism.

Table IV. *Partition of urinary N*

Period	Sheep	Total N g.	Urea N g.	NH ₃ N g.	Amino- acid N g.	Hip- puric acid N g.	Pre- formed crea- tinine g.	Crea- tinine g.	Uric acid g.	Purine base g.	Allan- toin g.
1 (low P)	1	12.77	8.09	1.77	0.21	0.33	1.44	0.64	0.32	0.39	6.45
	2	10.67	5.16	1.64	0.25	0.25	1.51	0.87	0.37	0.35	5.85
	3	12.99	8.04	1.12	0.51	0.36	1.26	0.79	0.36	0.40	6.15
	4	9.49	4.49	0.46	0.33	0.13	1.11	0.69	0.35	0.41	4.72
2 (P supplement)	1	9.06	4.15	1.73	0.61	0.23	1.72	0.53	0.38	0.43	2.72
	2	10.44	5.47	1.37	0.32	0.29	1.69	0.81	0.39	0.48	3.07
	3	8.47	4.63	0.90	0.19	0.40	1.53	0.89	0.41	0.36	2.55
	4	9.86	4.94	0.81	0.32	0.40	1.53	0.91	0.42	0.48	2.89
3 (Ca + P supplement)	1	10.50	3.54	3.44	0.87	0.30	1.16	0.34	0.26	0.72	4.42
	2	12.21	6.57	2.17	0.51	0.21	1.12	0.55	0.29	0.59	5.02
	3	11.02	6.24	1.09	0.18	0.46	1.37	0.73	0.34	0.64	4.70
	4	11.04	6.21	1.04	0.34	0.40	1.29	0.57	0.32	0.62	4.82
4 (low P)	1	13.10	7.62	0.27	0.49	0.50	1.66	0.83	0.40	0.29	5.84
	2	14.87	9.52	0.53	0.21	0.66	1.72	0.79	0.39	0.20	6.24
	3	11.58	5.80	0.41	0.33	0.54	1.38	0.59	0.39	0.52	5.25
	4	11.12	6.46	0.43	0.38	0.52	1.38	0.68	0.38	0.39	4.94

The present results confirm those found by Godden & Ray [1937]. The possibility that the excess urinary N is due to a diminished utilization of food energy must, of course, be borne in mind.

(ii) *Exogenous metabolism.* The total exogenous output is unaffected by any alteration in the diet (Table IV). In all periods this amounts to slightly more than 70 % of the total N. There are, however, considerable variations within each group. The highest value, i.e. in sheep 1, period 3, was due to faecal contamination of the urine. The observed variations in the amino-acid N output are normal for ruminants [Morris & Wright, 1933, 1, 2; Hutchinson & Morris, 1936, 1, 2].

The extremely low ammonia-N output in period 4 for all animals cannot at present be explained. This decrease is outside the normal range of variations and may possibly be the cumulative effect of periods 3 and 4, during both of which the Ca : P ratio was abnormal.

(iii) *Creatinine-creatinine metabolism.* The creatinine output, particularly when expressed as % of total N, is markedly higher in period 2 than in the remaining three periods. The lowest values occur in period 3.

The creatine output remains unchanged in total amount but the percentage is lower with the low P diets. In period 3 there is both a total and percentage decrease. This appears readily explicable, since P is intimately connected with the creatine metabolism, both varying in the same direction.

(iv) *Purin metabolism.* In the P-deficient periods both uric acid and purine base excretions, when expressed as % of total N, are lower than in the normal period. In period 3 the uric acid decreases but the purine base increases markedly.

The P deficiency causes a marked increase in the urinary output of allantoin, the diet with the abnormal Ca : P ratio having a less striking effect in the same direction. It is interesting to note that Terroine [1936] obtained similar results and claimed that this increase is due to synthesis of allantoin from ingested protein and is not due to an increased catabolism of body tissue.

(v) *Sulphur metabolism.* Details of the S metabolism are shown in Table V. In the P-deficient periods the urinary S output is greatly diminished with a correspondingly large increase in the N : S ratio. In period 3, although the S output remains constant (as compared with period 2) the N has increased, with a corresponding slight increase in the N : S ratio.

Table V. *S balance*

Period	Sheep	Urine N g.	Urine S g.	N : S ratio
1 (low P)	1	12.77	1.45	8.8
	2	10.67	1.47	7.3
	3	12.99	1.80	7.2
	4	9.49	0.99	9.6
2 (P supplement)	1	9.06	2.64	3.4
	2	10.44	2.73	3.8
	3	8.47	2.80	3.0
	4	9.86	2.69	3.7
3 (Ca + P supplement)	1	10.50	2.72	3.9
	2	12.21	2.71	4.5
	3	11.02	2.84	3.9
	4	11.04	2.52	4.4
4 (low P)	1	13.10	1.09	12.0
	2	14.87	1.28	11.6
	3	11.58	0.94	12.3
	4	11.12	1.10	10.1

To account for the diminished excretion of urinary S the possibility must be considered that, in a diet deficient in P, S may be able to replace P to some extent in the metabolic reactions in the body. No evidence on this point is, however, available.

Table VI. *S output in faeces*

Period	Sheep	Wt. dry faeces g.	N in faeces g.	S in faeces g.	S g. per kg. dry faeces	N : S of faeces
2	1	251.70	8.47	1.55	6.16	5.4
	2	293.00	8.19	1.54	5.25	5.3
	3	314.70	10.85	1.55	4.92	7.0
	4	281.50	8.18	1.66	5.89	4.9
	1	349.18	8.90	1.94	5.56	4.6
	2	315.85	7.05	1.89	5.98	3.8
	3	337.23	8.05	1.95	5.78	4.1
	4	330.00	7.51	1.88	5.60	4.0
4	1	156.67	7.39	0.93	5.93	7.9
	2	210.04	7.86	1.13	5.37	7.0
	3	197.30	7.71	1.03	5.22	7.5
	4	353.32	8.74	1.80	5.09	4.9
		Period		2	3	4
Mean S g. per kg. dry faeces of all 4 animals				5.56	5.75	5.40
Mean N g. per kg. dry faeces of all 4 animals				30.21	23.64	32.22

The faecal S excretion and the N:S ratio in the faeces are shown in Table VI. It will be seen that the S output varies directly with the dry weight of the faeces, and not, as in the case of the N output, with the dry matter of the ingested food. Further work on this subject has been carried out which shows that the result is definitely not due to any inherent error in the analytical technique used.

Nitrogen balance. The N balance (Table VII), although positive in each period, is markedly lower in periods 1 and 4 with a P-deficient diet. This agrees with the weight gains previously noted.

Table VII. *N balance and biological value*

Period	Sheep	N intake g.	N output g.	Daily balance g.	Biological value
1 (low P)	1	26.56	19.25	3.66	60
	2	28.16	18.58	4.79	72
	3	23.80	19.30	2.25	53
	4	26.56	16.12	5.22	72
2 (P supplement)	1	30.10	17.53	6.29	78
	2	30.10	18.63	5.74	76
	3	30.10	19.32	5.39	78
	4	30.10	18.04	6.03	75
3 (Ca + P supplement)	1	30.10	19.40	5.35	73
	2	30.10	19.26	5.42	70
	3	30.10	19.07	5.52	72
	4	30.10	18.55	5.78	72
4 (low P)	1	24.68	20.49	2.10	54
	2	26.24	22.73	1.76	53
	3	24.68	19.29	2.70	60
	4	24.68	19.86	2.41	60

The apparent biological value of the protein has been calculated. The formula used is the customary one

$$1 - \frac{U - E}{I - (F - M)},$$

where U = urinary N, E = endogenous urinary N, I = intake, F = total faecal N and M = metabolic faecal N. The figures for M and E were taken from the results of Hutchinson & Morris [1936, 2]. It is clear that an appreciably lower figure is

recorded with the P-deficient diets. The apparent biological value for period 3 is lower than that for period 2 and appears to substantiate the claim that in this period the results are transitional between those for the normal and the P-deficient periods.

The results of these determinations of the biological value are important in that they show the impossibility of calculating biological values from the results of experiments in which all food factors except the protein are not present in sufficient amount.

Nitrogen metabolism. A résumé of the results in the present experiment shows, therefore, that the exogenous metabolism remains unaltered during the feeding of a P-deficient diet. Since the endogenous metabolism is represented by the creatinine, creatine, purine and S metabolism, it would appear that, with a P-deficient ration, or with a ration containing an abnormally large Ca : P ratio, the endogenous metabolism is diminished. That this will eventually lead to active tissue catabolism appears probable from the work of Theiler, Du Toit and others. In the present instance, however, the excess urinary N found on feeding diets deficient in P is due to the ingested protein and is exogenous in origin. The excess allantoin must, therefore, also arise from the ingested protein, as Terroine has claimed.

The primary effect of a P-deficient diet is, therefore, a slowing-down of tissue formation with a consequent deamination and excretion of the excess of amino-acids. The decreased protein digestibility is probably the result of a diminished secretion of digestive juices and enzymes, which appear to use P in their metabolic reactions.

Mineral metabolism. The Ca and P balances are shown in Tables VIII and IX. With a P-deficient diet there was a loss of both Ca and P. When the supplement of P was added (period 2) a positive balance was recorded.

Whereas the urinary Ca increased in periods 1 and 4, the urinary P almost disappeared owing to the very low intake. In any period the amount of P excreted in the urine is only about 1 % of the total P excreted. The Ca excretion varies markedly. In the normal period 2 the Ca excretion in the urine is only about 0.5 % of the total output, whereas in the P-deficient periods it is about 5 %. In period 3, with the abnormal Ca : P ratio, the urinary Ca is about 1-2 %

Table VIII. *CaO balance*

Period	Sheep	CaO intake in 2 days g.	CaO output in 2 days			Daily balance g.
			Faeces g.	Urine g.	Total g.	
1 (low P)	1	10.78	10.88	0.46	11.34	- 0.28
	2	12.02	13.42	0.46	13.88	- 0.93
	3	9.50	11.14	0.60	11.74	- 1.12
	4	10.50	11.26	0.66	11.92	- 0.71
2 (P supplement)	1	13.80	12.04	0.08	12.12	+ 0.84
	2	13.80	12.60	0.06	12.66	+ 0.57
	3	13.80	12.70	0.04	12.74	+ 0.53
	4	13.80	11.92	0.06	11.98	+ 0.91
3 (P + Ca supplement)	1	128.04	118.92	2.56	121.48	+ 3.28
	2	128.04	119.08	1.76	120.84	+ 3.60
	3	128.04	122.76	1.50	124.26	+ 1.89
	4	128.04	124.52	1.16	125.68	+ 1.18
4 (low P)	1	9.02	11.50	0.56	12.06	- 1.52
	2	10.22	12.42	0.44	12.86	- 1.32
	3	9.02	11.40	0.68	12.08	- 1.53
	4	9.02	11.40	0.46	11.86	- 1.42

Table IX. P_2O_5 balance

Period	Sheep	P_2O_5 intake in 2 days g.	P_2O_5 output in 2 days			Daily balance g.
			Faeces g.	Urine g.	Total g.	
1 (low P)	1	2.22	2.54	0.02	2.56	- 0.17
	2	2.24	3.52	0.02	3.54	- 0.65
	3	1.90	3.70	0.02	3.72	- 0.91
	4	2.08	3.26	0.02	3.28	- 1.10
2 (P supplement)	1	12.86	12.42	0.10	12.52	+ 0.17
	2	12.86	11.30	0.16	11.46	+ 0.70
	3	12.86	11.68	0.60	11.74	+ 0.56
	4	12.86	11.04	0.20	11.24	+ 0.81
3 (P + Ca supplement)	1	12.86	10.96	0.11	11.07	+ 1.79
	2	12.86	12.86	0.11	12.97	- 0.11
	3	12.86	13.45	0.05	13.50	- 0.64
	4	12.86	13.19	0.04	13.23	- 0.37
4 (low P)	1	1.58	3.14	0.02	3.16	- 0.79
	2	1.84	3.78	0.02	3.80	- 0.98
	3	1.58	3.58	0.00	3.58	- 1.00
	4	1.58	3.30	0.02	3.32	- 0.87

of the total. This agrees with the theory of Cayla [1935] that an excess of Ca over P in the diet may lead, whether the P intake is deficient or not, to a deficiency of both Ca and P. This may possibly be due to a precipitation of insoluble Ca salts in the alimentary tract, whereby they are not absorbed and utilized.

SUMMARY

1. A complete study has been made of the effect of a P-deficient diet on the protein and mineral metabolism of sheep.

2. An increased output of urinary N was noted in the periods of P deficiency and when a diet with an abnormally high Ca : P ratio was fed, although the food intake was lower.

3. The exogenous N metabolism was unaffected by a P-deficient diet, but the endogenous metabolism was diminished.

4. In the P-deficient periods there were losses of both Ca and P, which were remedied by the addition of a supplement of P.

5. The proportion of the total Ca excreted found in the urine rose, in the P-deficient periods, from 0.5 to 5%.

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CXLIX. THE FASTING METABOLISM OF RUMINANTS

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IN a previous publication [Hutchinson & Morris, 1936], a short study was presented of the fasting catabolism of ruminants, together with a brief review of the more important studies up to that date. The present experiments were undertaken to obtain a more detailed analysis of the protein catabolism by means of systematic and complete analyses of both blood and urine. With the ruminant, most of the evidence in connexion with protein anabolism and catabolism has been obtained from experiments on feeding with normal or abnormal rations [Morris *et al.* 1936]. To understand completely the processes concerned, a study of the organism when living at the expense of its own tissues is essential. With water available the fasting animal obtains the energy necessary for the continuation of the life processes from the catabolism of its own store of carbohydrate, protein and fat.

The animals used in the present experiment were goats (2 lactating, 2 non-lactating), sheep (4 non-lactating) and cows (3 lactating). The animals fasted following a ration complete in all respects for their requirements. In the previous experiments [Hutchinson & Morris, 1936; Benedict & Ritzman, 1927] the animals were subjected to a period of low protein intake prior to starvation. In view of the statement by Aylward & Blackwood [1936] that demineralization of the osseous tissue takes place during fasting, the mineral (Ca and P) metabolism was studied in the case of the goats and sheep.

EXPERIMENTAL

During the pre- and post-fast periods collections of urine and faeces were made every 2nd day; during starvation daily collections and analyses were carried out. The lactating animals were milked twice daily, the evening and morning milk being bulked for analysis; with the goats the yield fell so low that only total N analyses could be made. So remarkable, with the goats, was the rise in N percentage in the milk that it was thought advisable, with the cows, to obtain a more detailed analysis of the nitrogenous products of the milk throughout the fast. A study was made of the casein, albumin, globulin and N.P.N. of the milk before, during and following the fast, though the results are not presented here.

Goat 2 died at the end of the 10-day fast, the urinary volume being abnormally high. Cow 1 suffered from "milk fever" on the 3rd day of the fast, but recovered within a few hours after udder inflation. From the results it is doubtful if the "milk fever", apart from 1 day, had any effect on the protein or mineral metabolism.

The methods used in the analyses were as follows:

Urine. Total N, Kjeldahl; urea and ammonia, Folin aeration; amino-acid N, Sørensen; creatinine, Jaffe; uric acid, Folin; purine base, Krüger-Schmidt; allantoin, Roy; Ca, McCrudden; P, Pemberton-Neumann.

Faeces. Total N, Kjeldahl; Ca, permanganate titration; P, Pemberton-Neumann.

Blood. Total N, Kjeldahl; serum albumin and globulin, Howe; amino-acid, Folin; N.P.N., micro-Kjeldahl; uric acid, Folin; Ca, Collip; P and phosphatase, Bodansky; sugar, Hagedorn-Jensen; Cl, Davies.

In the discussion of results the paper is divided into two parts:

1. Nitrogen metabolism:
 - (a) Urinary N partition.
 - (b) Faecal N excretion.
 - (c) Blood N partition.
2. Mineral metabolism:
 - (a) Ca excretion in urine and faeces.
 - (b) P excretion in urine and faeces.

DISCUSSION OF RESULTS

1. Nitrogen metabolism

(a) *Urinary N partition.* In the previous publication [Hutchinson & Morris, 1936] it was shown that, following a low N diet, the most marked effects on the urinary N partition were the increases in urea and creatine excretion. The ammonia output tended to fall off until the 8th day of fast when an increase above normal was noted. In all cases the urinary N excretion increased markedly during the fast, the true fasting catabolism being reached on the 4th to the 6th day, thus confirming the earlier work of Carpenter [1927]. The daily N excretion per kg. body-weight was found to be 0.088 to 0.13 g. for the cows, 0.13 g. for the goats and 0.16 g. for the sheep.

Table I shows the results in the present experiment. The total N excretion tends to decrease slightly as the fast continues. It must again be stressed that the fast followed a rich and complete diet for several months. The N excretion expressed as g. per kg. body-weight is as follows:

Goat				Sheep				Cow	
1	2	3	4	1	2	3	4	1	2
0.20	0.12	0.16	0.17	0.12	0.17	0.16	0.16	0.06	0.08

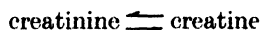
With the cows and sheep the results are slightly lower than in the previous experiment, whereas with the goats the results are higher. These differences would appear to be within the normal range and dependent on the age and state of nutrition of the animal prior to the fast.

Regarding the urinary partition, the urea, ammonia, amino-acid and hippuric acid excretions will be discussed first. With the goats, both the urea and ammonia N excretions when expressed as a percentage of the total N, increase as the fast progresses, whereas the hippuric acid N output decreases markedly. The amino-acid N excretion remains fairly normal. With the cows, only the urea increases, the ammonia, amino-acids and hippuric acid falling off markedly during the fast. With the sheep, both urea and ammonia increase, the amino-acids and hippuric acid decreasing. It will be seen that the percentage output follows closely the total output of these partition products. Comparing these results with the few reported in previous publications [Hutchinson & Morris, 1936; Carpenter, 1927], it will be noted that, on broad lines, both series of experiments are in agreement.

Table I. *Urinary partition of N*

Day of fast	Total N g.	Urea N g.	NH ₃ N mg.	Amino acid N mg.	Hippuric acid N mg.	Preformed creatinine mg.	Creatine mg.	Uric acid mg.	Purine base mg.	Allantoin g.
Goat 1										
Non-fast	4.84	3.39	160	110	530	310	100	70	150	0.89
1- 3	4.71	3.37	217	37	190	350	320	73	113	1.23
4- 6	3.68	2.74	290	90	53	340	63	60	237	0.66
7- 9	5.03	3.84	407	157	40	267	380	37	320	0.68
10	7.72	6.36	650	110	60	370	250	30	490	0.59
Post-fast	4.20	2.04	920	250	430	240	90	60	170	0.74
Goat 2										
Non-fast	4.50	3.14	150	100	510	360	130	60	140	0.89
1- 3	4.54	3.70	53	37	190	300	153	37	230	0.86
4- 6	3.57	2.56	130	57	50	373	277	40	200	0.62
7- 9	3.43	2.61	303	37	50	307	237	40	260	0.63
10	7.56	5.88	450	220	20	870	410	90	590	1.10
Goat 3 (lactating)										
Non-fast	6.18	3.50	190	40	1280	300	120	170	320	1.76
1- 4	7.69	5.01	263	165	1123	370	335	193	598	2.06
5- 8	5.86	4.83	245	155	133	305	230	63	345	0.50
9-12	4.12	2.95	548	83	20	298	123	45	295	0.51
Post-fast	5.28	2.79	390	230	820	260	90	120	230	1.67
Goat 4 (lactating)										
Non-fast	6.08	3.24	200	100	1100	280	150	150	500	1.55
1- 4	6.49	4.87	75	85	485	300	203	70	503	1.81
5- 8	5.42	4.26	150	95	193	348	243	78	595	0.73
9-12	5.31	4.01	278	78	75	340	333	80	520	0.79
Post-fast	4.60	1.73	480	230	860	200	60	80	240	1.80
Sheep 1										
1- 3	8.58	5.98	140	90	150	570	347	127	290	2.74
4- 5	6.49	4.65	285	50	70	690	135	150	415	1.70
6- 7	5.53	3.90	520	45	55	560	225	195	525	1.34
Post-fast	6.39	4.04	890	110	170	720	320	160	200	3.23
Sheep 2										
1- 3	7.34	4.87	153	233	187	640	513	113	310	2.61
4- 5	6.14	4.31	190	55	85	610	650	140	365	1.80
6- 7	7.86	5.80	415	115	135	845	350	270	560	1.85
Post-fast	5.34	2.58	820	130	130	760	440	180	180	2.93
Sheep 3										
1- 3	6.97	4.31	203	57	460	500	463	93	270	2.01
4- 5	8.64	6.25	640	80	80	840	430	60	360	1.22
6- 7	8.65	6.33	275	35	125	730	315	280	525	2.17
Post-fast	6.50	4.02	560	260	180	630	400	180	200	3.08
Sheep 4										
1- 3	8.65	6.58	177	87	173	580	260	160	410	2.83
4- 5	6.48	4.82	240	35	85	615	485	155	490	1.72
6- 7	5.97	4.53	375	40	10	580	265	185	610	1.40
Post-fast	4.50	2.50	230	170	70	560	350	180	200	2.36
Cow 1 (lactating)										
			g.	g.	g.	g.	g.	g.	g.	
Non-fast	39.32	13.96	9.48	4.04	2.18	3.56	0.74	0.71	1.83	11.57
1- 3	44.39	20.12	16.30	4.48	0.53	2.78	2.31	0.39	1.44	16.67
4- 6	37.26	7.77	20.99	3.57	0.25	2.57	1.81	0.22	1.11	7.99
7- 9	21.80	10.31	6.00	1.21	0.23	1.39	0.99	0.13	0.54	6.38
10-12	24.75	13.65	4.46	1.26	0.21	2.54	1.60	0.19	0.93	5.86
Post-fast	36.05	9.15	9.15	3.90	2.27	3.64	0.42	0.93	3.30	18.38
Cow 2 (lactating)										
Non-fast	37.28	14.30	11.95	3.85	2.61	4.08	0.74	0.71	1.83	11.57
1- 3	64.40	17.05	31.04	5.13	1.05	3.09	3.83	0.69	1.15	18.57
4- 6	36.15	19.49	7.51	2.90	1.13	4.02	2.47	0.30	0.81	4.78
7- 9	37.75	25.92	4.37	1.54	0.82	4.47	1.86	0.33	0.33	4.78
10-12	31.86	20.87	3.63	1.10	0.34	2.84	0.85	0.24	0.45	5.29
Post-fast	40.04	15.47	10.75	4.25	0.23	4.05	1.43	1.00	2.47	9.34

Regarding the creatinine and creatine metabolism (Table I), two points are evident, the normal or slightly subnormal value of the creatinine output and the large increase in the creatine excretion. The percentage creatinine N tends to increase slightly towards the 6th day of fast and then decreases. It is doubtful if the subnormal results are significant, since fairly large variations in the creatinine excretion are to be expected normally. Previous work on human subjects [Cathcart, 1921] has shown that there is a tendency towards a decreased output of both creatinine and creatine as fasting progresses. It is remarkable that only with the animal which died is there any marked increase in the creatinine output in the urine, corresponding with a marked increase in the urinary volume. From the complete correlation between urinary volume and creatinine excretion a hypothesis could be formulated regarding the formation of creatinine. A reversible enzyme reaction, such as



inhibited by the production of a relatively excessive amount of creatinine, would explain the constant excretion. The only condition which would permit of an excessive formation and excretion of creatinine would be one in which there was a marked diuresis with a consequent rapid and continuous flushing of the tissues, as in the case of goat 2. This would agree with the theory of Terroine & Champagne [1933] that creatinine metabolism is the final stage of some fundamental process.

It has been stated [Terroine & Mourot, 1931] that creatine is formed either from ingested protein, on feeding a protein-rich diet, or during starvation from body tissue. The former assertion, the increase in creatine excretion with an increased protein intake, we have not yet been able to confirm with ruminants [Hutchinson & Morris, 1936]; further work is, however, in progress to study this problem more fully. The increased creatine output during starvation has long been known, and it is reasonable to assume that this arises from active muscular catabolism. Whether the increase is merely the result of the protein catabolism or is due to the catabolism of some substance, a precursor of creatine but not connected directly with the true protein metabolism, is at present unknown.

The purine metabolism is shown in Tables I and II. As in the non-ruminants, the uric acid excretion decreases markedly both in total and percentage amount, with, as will be seen later, a marked increase in the blood level. The purine base compounds increase, both in total amount and when expressed as a percentage of the total N, with the goats and sheep, especially the non-lactating ones; with the cows a decrease is noted. With all the animals a decrease occurs in the allantoin excretion following an initial rise in the first 2 days. It is evident that the amount of the total N excreted as purine base metabolites has diminished considerably during starvation. The ruminant thus appears to reduce nuclear cell metabolism to a minimum in order to preserve the nucleoproteins which are essential for life processes. This fact was noted by Hutchinson & Morris [1936], who found that the percentage of total N in the urine present as undetermined N was reduced. They stated that "this fraction consists of the purine bodies, and it appears that the ruminant tends to retain these compounds by minimizing cellular metabolism during a fast". This agrees also with the results obtained with non-ruminants [Benedict, 1907; 1915; Cathcart, 1921]. It is, however, possible that a part of the purine bodies excreted arises by synthesis from non-purine material. It has been found that, whereas a fat-rich protein-poor diet decreases the purine output, a carbohydrate-rich diet causes a marked increase

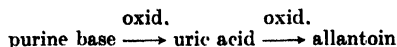
Table II. *Percentage distribution of purine N*

	Days of fast													Post-
	Pre-	1	2	3	4	5	6	7	8	9	10	11	12	fast
Goat 1														
Uric acid	6	5	5	4	6	—	6	7	3	2	2	—	—	5
Purine base	12	5	10	10	19	—	35	32	32	28	44	—	—	16
Allantoin	82	90	85	86	75	—	59	61	65	70	54	—	—	79
Goat 2														
Uric acid	5	4	3	2	5	—	3	4	3	4	5	—	—	—
Purine base	12	16	17	25	22	—	22	23	30	28	32	—	—	—
Allantoin	83	80	80	73	73	—	75	73	67	68	63	—	—	—
Goat 3														
Uric acid	7	7	—	7	—	6	8	9	7	5	4	3	—	6
Purine base	12	11	—	14	—	36	35	39	18	32	24	32	—	10
Allantoin	81	82	—	79	—	58	57	52	75	63	72	65	—	84
Goat 4														
Uric acid	6	3	—	2	—	6	6	7	5	6	7	6	—	3
Purine base	22	21	—	18	—	44	23	35	43	30	35	30	—	11
Allantoin	72	76	—	80	—	50	71	58	52	64	58	64	—	86
Sheep 1														
Uric acid	—	1	2	7	6	6	10	8	—	—	—	—	—	—
Purine base	—	6	14	8	19	16	15	32	—	—	—	—	—	—
Allantoin	—	93	84	85	75	78	75	60	—	—	—	—	—	—
Sheep 2														
Uric acid	—	3	2	7	7	6	10	9	—	—	—	—	—	—
Purine base	—	10	10	8	19	11	19	21	—	—	—	—	—	—
Allantoin	—	87	88	85	74	83	71	70	—	—	—	—	—	—
Sheep 3														
Uric acid	—	4	1	7	—	4	8	10	—	—	—	—	—	—
Purine base	—	11	17	6	—	21	15	20	—	—	—	—	—	—
Allantoin	—	85	82	87	—	75	77	70	—	—	—	—	—	—
Sheep 4														
Uric acid	—	3	2	7	6	6	8	7	—	—	—	—	—	—
Purine base	—	8	16	13	19	23	18	33	—	—	—	—	—	—
Allantoin	—	89	82	80	75	71	74	60	—	—	—	—	—	—
Cow 1														
Uric acid	5	2	2	2	2	2	2	2	3	2	5	2	3	4
Purine base	12	6	19	10	9	16	10	8	9	6	19	13	15	14
Allantoin	83	92	89	88	89	82	88	90	88	92	76	85	82	82
Cow 2														
Uric acid	5	4	4	3	4	4	5	7	7	6	5	3	3	—
Purine base	12	6	4	6	16	14	11	5	6	4	8	5	7	—
Allantoin	83	90	92	91	80	82	84	88	87	90	87	92	90	—

[Catheart, 1909; Graham & Poulton, 1913; Umeda, 1915]. Terroine & Mouroit [1931] noted a direct correlation between the level of protein intake and the allantoin output in the urine. This may possibly be due to the presence in the protein molecule of amino-acids capable of being synthesized to purine compounds [Ackroyd & Hopkins, 1916; Lewis & Doisy, 1918]. Since, however, purine synthesis appears to be connected with carbohydrate metabolism, it can be safely concluded that during starvation, with a lack of carbohydrate, this synthesis can only take place to a very limited extent.

Examination of the distribution of the purine N (Table II) shows that the percentage distribution has been markedly altered throughout starvation. With

the goats the normal proportions of purine base, uric acid and allantoin are approximately 12 : 6 : 82, whereas in starvation they are 32 : 3 : 65; with the sheep the normal values 11 : 5 : 83 become 30 : 8 : 62. These results show a marked increase in purine base and as marked a decrease in allantoin. According to the commonly accepted theory of purine metabolism



there must have been a decrease both in the cellular oxidation of the nucleoproteins and in the oxidation of the purine base compounds formed during starvation by goats and sheep. With the cows, on the other hand, it is doubtful if the changes are significant. This is not the effect of lactation, since there is little difference between the lactating and non-lactating goats, but must be a species difference.

Although no difference is found between the lactating and non-lactating goats during starvation, it is interesting to compare the results obtained in the pre-fast period. The total amount of purine base derivatives is greater with the lactating animals. This increased cellular oxidation may be due both to the increased food intake and to a natural increase as the result of lactation.

It seems advisable at this stage to draw attention to the figures given by Hunter & Givens [1914], and now generally accepted, for the purine base distribution in the urine of goats, cows and sheep, and to compare them with the present values. It will be seen that, whereas Hunter & Givens found markedly different distributions for goats, sheep and cows, the present work indicates that there are, in fact, no significant differences in distribution.

	Goat		Sheep		Cow	
	H. and G.	Present work	H. and G.	Present work	H. and G.	Present work
Uric acid	7	6	16	5	7.3	5
Purine base	12	12	20	11	0.7	13
Allantoin	81	82	64	83	92	82

The sulphur excretion and the N : S ratio of the urine (Table III) show that a difference exists between the different species of ruminant. Whereas the goats and cows, towards the latter end of the fast, show a N : S ratio approximating closely to that of body tissue, the sheep show a ratio higher than that of body tissue, due to a marked retention of S. This may be due, at least in part, to the excessive requirements of S for wool production with sheep, and the attempt by the animals to retain as much body S as possible. The sheep were growing and this may also have had some effect on the ability of the animals to retain body S. It is possible, however, that the muscle or reserve protein catabolized in the course of the first 7 days' starvation is poor in S. Similar results were noted by Lewis [1916], who found a slight retention of S indicating the possibility of a retention of muscle tissue.

The N : P ratio (Table IV) falls rapidly as the fast progresses, from 350 to 17.5 with goat 1, to 6.5 with goat 2, to 21 with goat 3 and to 22 with goat 4. With the 4 sheep the ratio falls from 350 to about 18. With human beings [Cathcart, 1921] the ratio reaches a maximum after 12 to 14 days of about 6, the theoretical value for muscle being 6.6. Cathcart concludes that the bony tissue contributes materially to the P excretion in starvation, since the excretion of P in its relation to N is greater than the ratio of the two elements in muscle. Aylward & Blackwood [1936] came to the same conclusion with ruminants. They found that the N : P ratio towards the end of the fast was 11.6 instead of

Table III. *N-S ratios*

Day of fast	Goat				Sheep				Cow	
	1	2	3	4	1	2	3	4	1	2
Pre-fast	3.7	3.9	4.4	4.8	3.7	7.6	5.5	5.8	3.5	—
1	14.0	10.3	12.5	14.0	23.6	17.2	15.9	23.2	9.4	11.5
2	15.0	16.1	—	—	22.1	25.2	21.1	22.2	14.3	13.9
3	9.0	17.4	—	—	49.0	32.8	28.5	30.4	15.4	12.3
4	10.9	17.1	15.5	16.3	40.6	34.5	—	45.2	14.0	11.5
5	—	—	16.1	14.2	31.5	24.2	23.4	33.0	17.8	10.6
6	10.6	15.6	14.3	17.4	33.7	21.1	23.4	33.6	14.3	13.9
7	9.6	16.2	15.5	16.2	30.0	20.8	18.3	29.2	15.1	15.4
8	12.2	15.3	16.1	15.3	—	—	—	—	14.2	18.8
9	13.6	13.9	14.9	16.5	—	—	—	—	16.6	19.1
10	14.4	14.0	17.2	13.3	—	—	—	—	12.9	17.6
11	—	—	15.4	16.2	—	—	—	—	14.9	18.1
12	—	—	15.7	16.3	—	—	—	—	11.6	16.9
Post-fast	4.4	—	—	—	—	—	—	—	—	—

Table IV. *N-P ratios*

Day of fast	Goat				Sheep			
	1	2	3	4	1	2	3	4
Pre-fast	345	350	258	320	345	236	255	551
1	—	—	—	—	360	320	248	287
2	87.7	63.5	91	95	255	229	310	300
3	—	—	—	—	430	126	273	170
4	—	—	—	—	105	40	542	52
5	11.0	8.4	56	51	19.2	24	—	38
6	—	—	—	—	19.5	15	29	24
7	17.5	6.5	22	23	16.9	15	18	20
8	—	—	—	—	16.0	17	24	15
9	—	—	21	22	—	—	—	—

17.4, and state that bone demineralization must take place to account for the decrease in the ratio. In the present experiment no such assumption is necessary, since the P arising from the catabolism of muscle can account for all the urinary P excreted. From the results with goat 2, which died at the end of the fast, it would appear, more especially from the $N:P$ and $N:S$ ratios, that the last 3 days of the fast correspond to the immediate premortal stage in the life of the animal. With goat 1 the ratio of $N:P$ fell markedly from the 4th to the 7th day of the fast, as did also the $N:S$ ratios. This abnormal output of P and S cannot at present be explained, but is obviously the result of excessive catabolism of some tissue rich in P and S.

It is doubtful if the daily variations in the $N:P$ and $N:S$ ratios are significant; it may be advisable to average the ratios from the 4th day of fasting. In the present experiment this would still yield ratios of 17 or more, which correspond to those found in the experiments of Aylward & Blackwood [1936].

(b) *Faecal N excretion.* In a previous publication [Hutchinson & Morris, 1936] the constant fraction of the metabolic faecal N of cows was determined. The average value was 7 g. daily. In the present investigation values have been obtained for goats, cows and sheep (Table V). From the 4th day the faecal N remained fairly constant, except with the sheep. The average values of the daily faecal N output are, from the 4th to the last day of fast,

Goat				Sheep				Cow	
1	2	3	4	1	2	3	4	1	2
0.39	0.44	0.35	0.50	0.49	0.49	0.88	0.68	6.7	6.6

Table V. *Faecal N output, g.*

Day of fast	Goat				Sheep				Cow	
	1	2	3	4	1	2	3	4	1	2
Pre-fast	4.02	2.95	5.33	5.59	7.13	8.82	8.03	6.79	93.27	96.40
1	2.08	2.20	6.20	5.98	1.63	3.26	3.26	1.69	52.90	44.50
2	1.45	0.85	—	—	0.72	1.00	0.70	0.57	33.60	27.50
3	1.04	0.64	2.90	2.24	1.07	0.67	0.97	0.96	10.58	16.30
4	1.33	1.09	—	—	0.44	0.18	—	0.32	—	15.37
5	—	—	0.78	0.42	0.61	0.65	—	0.95	19.24	16.68
6	—	0.76	1.00	0.68	0.56	0.47	1.97	0.91	5.88	11.03
7	0.37	—	0.58	0.60	0.34	—	1.62	0.36	11.74	12.60
8	—	—	0.28	0.75	—	—	—	—	—	8.11
9	0.66	0.60	0.26	0.50	—	—	—	—	5.07	6.28
10	—	—	0.28	0.24	—	—	—	—	2.96	5.94
11	—	—	0.38	0.45	—	—	—	—	—	6.18
12	—	—	0.32	0.33	—	—	—	—	11.85	—
Post-fast	3.42	—	5.58	5.08	—	—	—	—	62.70	64.28

The average works out at 0.42 g. for goats, 0.64 g. for sheep and 6.5 g. for cows. The value for the sheep is higher than would be expected, but this is probably the result of the short fast (7 days) as compared with 10 and 12 days for the goats and cows. It will be seen that the results for the cows agree with those previously recorded. From work on a fasting dog, Voit [1894] noted that the ordinary starvation faeces cannot consist of the total of the excretions from the body into the alimentary tract, but rather represent their unabsorbed remainder. In the human subject, Benedict [1915] found that during a fast of 31 days no faeces were excreted at all.

(c) *Blood N partition.* The blood analyses (Table VI) show an increase in both the protein and non-protein fractions. The apparent increase in total protein, although in some cases slight, is nevertheless definite and significant. It is, however, probably not the result of a true increase but rather due to a slight dehydration of the blood. This is borne out by an examination of the change occurring in the corpuscular volume during starvation. A marked increase is noted in the case of the cows (Table VII). In the dog and rat no such increase in corpuscular volume was found [Robertson, 1912-13].

Analysis of the protein partition shows that both albumin and globulin increase to a varying degree. In both goats and sheep the globulin increases to more than 50% of the total protein, whereas in the cow the increase is very much smaller, the albumin increasing to a greater extent. Lewinski [1903] found an increase in the globulin fraction in a fasting dog but no change in the albumin. Robertson [1912-13] found that in the dog and rat starvation led to an increase in the globulins, whereas in the rabbit, ox and horse the albumins increased.

The increase in urea and amino-acid N may also be explained by dehydration. On the other hand, the increase in the undetermined fraction of the N.P.N. cannot be due to dehydration. It is this fraction, with the goats and cows, which causes the increase in the N.P.N. The nature of this fraction is at present unknown. It may be noted that, with the sheep, the undetermined N is higher in the post-fast period than during starvation. This may possibly be the result of the low P ration on which the animals subsisted both before and after the fast.

With all animals the uric acid increases markedly. Similar results have been recorded with human beings [Cathcart, 1921], in whom uric acid is the chief end product of the purine catabolism.

The blood sugar and Cl results are also included (Table VII). With cow 1, at the height of the "milk fever", the blood sugar rose markedly. During the

Table VI. *Blood N partition*

Period	Total protein g.	Albumin g.	Globulin g.	N.P.N. mg.	Urea N mg.	Amino-acid N mg.	Uric acid mg.	Undetermined N.P.N. mg.
Goat 1								
Pre-fast	6.00	3.40	2.60	27.00	13.04	5.84	-	8.12
Fast	6.76	3.72	3.04	35.80	21.00	6.28	-	8.52
Post-fast	6.09	3.24	2.75	31.00	14.88	5.80		10.32
Goat 2								
Pre-fast	6.43	3.32	3.11	27.00	16.62	6.02	--	4.36
Fast	7.13	3.58	3.55	37.60	25.02	5.98		6.60
Goat 3								
Pre-fast	5.90	3.08	2.82	21.80	14.82	5.02		1.96
Fast	6.54	3.17	3.37	28.20	18.94	5.88		3.38
Post-fast	6.08	3.12	2.96	22.22	16.42	5.56		0.24
Goat 4								
Pre-fast	6.69	3.38	3.31	19.60	11.68	4.84		3.08
Fast	7.27	3.42	3.85	23.60	16.04	5.02		2.54
Post-fast	6.35	3.26	3.09	17.70	12.84	4.78		0.08
Sheep 1								
Fast	6.14	3.08	3.06	32.20	22.02	7.44	0.08	2.74
Post-fast	6.08	3.00	3.08	27.00	18.40	5.48	0.07	3.12
Sheep 2								
Fast	6.66	3.28	3.38	34.70	24.00	7.76	0.12	2.94
Post-fast	6.22	3.30	2.92	31.00	19.20	5.14	0.09	6.66
Sheep 3								
Fast	6.46	3.19	3.27	34.10	23.66	7.88	0.08	2.56
Post-fast	6.25	3.21	3.04	32.00	19.70	5.64	0.07	6.66
Sheep 4								
Fast	6.58	3.22	3.36	35.20	24.02	7.38	0.10	3.80
Post-fast	5.93	3.10	2.83	32.00	18.80	5.66	0.07	7.54
Cow 1								
Pre-fast		4.13		15.68	7.94	6.69	-	1.05
	7.89	4.19	3.70	19.60	8.00	6.44		5.16
	9.44*	5.47	3.97	24.50	8.92	7.07		8.51
Fast	8.22	4.96	3.26	24.10	11.33	6.30	-	6.47
	8.17	4.86	3.31	32.20	9.70	7.56	0.12	14.94
	8.21	4.84	3.37	24.00	13.00	7.21	-	3.19
Post-fast	7.61	4.05	3.56	23.80	9.50	6.65		7.65
	8.06	4.74	3.32	17.80	10.44	6.88		0.48
Cow 2								
Pre-fast	7.50	4.34	3.16	17.68	8.24	7.07		2.37
	7.60	4.62	2.98	19.20	8.00	6.37	-	4.83
	7.93	4.78	3.15	19.80*	9.02	6.44	-	4.34
Fast	8.06	5.12	2.94	22.10	9.00	6.93	0.05	6.17
	8.50	5.21	3.29	28.70	10.28	7.42	0.05	11.00
	8.35	5.02	3.33	31.90	11.73	7.46	0.14	12.71
Post-fast	7.21	4.48	2.73	22.40	10.70	6.64	-	5.06
	7.93	4.78	3.15	17.91	11.00	6.86	-	0.05

* Milk fever.

Table VII. *Blood analyses*

Period	Corpus- cular vol. %	Ca mg./ 100 ml.	Inorganic P mg./ 100 ml.	Phos- phatase units	Cl mg./100 ml.		Sugar mg./100 ml.	
					Blood	Plasma	Blood	Plasma
Goat 1								
Pre-fast	—	9.1	5.4	8.5				
Fast	—	8.2	6.9	7.6				
Post-fast	—	9.2	4.8	8.6				
Goat 2								
Pre-fast	—	8.5	6.4	7.7				
Fast	—	8.0	12.0	3.5				
Goat 3								
Pre-fast	—	9.8	5.5	6.7				
Fast	—	8.1	7.2	4.2				
Post-fast	—	9.7	6.0	5.7				
Goat 4								
Pre-fast	—	10.4	4.9	6.9				
Fast	—	8.6	6.9	4.8				
Post-fast	—	9.4	5.9	7.0				
Sheep 1								
Fast	—	9.9	9.2	5.0				
Post-fast	—	13.4	2.7	12.8				
Sheep 2								
Fast	—	10.7	10.0	5.3				
Post-fast	—	12.2	3.1	11.7				
Sheep 3								
Fast	—	10.0	11.0	4.7				
Post-fast	—	13.6	2.2	11.4				
Sheep 4								
Fast	—	10.5	9.7	5.5				
Post-fast	—	14.1	2.4	13.0				
Cow 1								
Pre-fast	33.3	8.4	—	—	303	359	41.8	53.6
	34.0	—	—	—	298	360	39.1	54.2
	33.3	8.6	5.8	5.0	306	355	46.7	53.8
Fast	43.0*	5.8	4.3	3.9	271	343	147.5	208.0
	40.0	7.9	7.4	5.7	283	338	52.2	65.7
	38.0	7.5	5.2	5.5	282	334	56.4	68.1
	36.0	7.8	5.4	6.0	272	323	57.2	71.6
Post-fast	29.0	8.6	5.3	4.7	305	357	67.4	78.6
	30.0	9.8	4.3	5.4	301	360	55.2	58.7
	31.0	—	—	—	301	357	52.7	67.7
Cow 2								
Pre-fast	33.3	10.2	5.7	4.5	305	363	46.8	55.8
	32.5	10.6	5.1	4.7	306	368	44.5	53.6
	31.1	10.5	5.0	5.0	279	346	40.0	48.3
Fast	40.0	9.8	5.9	3.0	275	347	47.3	63.2
	41.0	10.2	6.5	4.1	267	337	48.7	67.8
	42.0	8.1	7.2	3.2	267	323	51.2†	71.5
Post-fast	33.3	10.6	4.6	4.8	296	365	45.0	58.4
	32.0	10.8	4.6	4.2	307	375	66.7†	87.0
	35.0	—	—	—	287	352	51.6	64.3

* Milk fever.

† Great difficulty in obtaining blood sample.

course of normal starvation, however, there may be a tendency towards a rise in blood sugar, but the significance of the results is doubtful. The effect of excitement on the blood sugar level has long been known and is well exemplified with cow 2, where an increase was noted when blood samples were obtained only with great difficulty. Aylward & Blackwood [1936] found no change in the blood sugar level of fasting cows. In non-ruminants glucose is present in the blood up to the last day of life, being produced, probably, from the metabolized protein [Lusk, 1931].

The Cl content of both whole blood and plasma decreases slightly during starvation. In non-ruminants plasma Cl decreases during starvation [Gamble *et al.* 1923; Goto, 1923; Weeks *et al.* 1923], the reduction being due, in part, to migration of Cl from plasma to cells as a result of fasting acidosis [Peters & Van Slyke, 1931]. With ruminants no change was noted in the corpuscular concentration of Cl.

2. Mineral metabolism

(a) *Ca excretion in urine and faeces.* With the progress of the fast the urinary Ca decreased markedly (Table VIII), and, with the sheep, almost disappeared. The faecal excretion of Ca fell steadily throughout the fast. With the goats the percentage faecal Ca in the dry faeces rose during the fast, although the total output fell, whereas with the sheep both the percentage and total output decreased. During the pre-fast period all the animals were in positive Ca balance.

(b) *P excretion in urine and faeces.* With the goats and sheep the urinary P rose rapidly immediately starvation began (Table VIII). With cows Aylward & Blackwood [1936] found that the pre-fast level was maintained for the first 4 days of starvation, a rise in urinary P excretion beginning on the 5th day. It has already been shown that the urinary P during starvation in ruminants can be accounted for by the protein catabolized. The faecal P output, on the other hand, fell markedly, at times to an amazingly low figure (sheep 1, 2 and 4). With both goat 1 and sheep 3 the increase in P excretion towards the end of the fast may be due, in part, to the voiding of very soft faeces in large amounts. It will be seen (Table V) that the faecal N excretion was extremely high on those days. During those days the percentage of P in the dry faeces, which had been falling markedly, rose to a very high level. With the other animals the percentage fell steadily throughout starvation. In the pre-fast period the sheep were in negative P balance, owing to a P deficiency in the diet. With cows, Aylward & Blackwood [1936] found that there was a marked increase in the P percentage in the faeces during starvation.

In the ruminant the contents of the digestive tract are never completely removed even after 8 days' starvation [Grouven, 1864]. Thus we have a state of minimum food intake rather than true starvation. In accounting for the Ca and P in the faeces this fact must be considered. Whereas in the non-ruminant during starvation any faecal Ca and P must be endogenous in origin, it is reasonable, with ruminants, to assume that a part at least will come from the food residues. This view finds support in the constancy of the Ca : P ratio in the pre-fasting and fasting faeces of the goats and sheep (Table IX). Further evidence on this point can be found in the results of Turner *et al.* [1934]. They found that in ruminants considerable amounts of Ca and P may be retained as insoluble compounds in the intestinal tract for several weeks.

In view of these facts it seems that the assumption made by Aylward & Blackwood [1936], that the faecal P and Ca are entirely endogenous in origin, must be fallacious. The possibility of a part of the Ca and P excretion being

Table VIII. *Ca and P output, g.*

Day of fast	Goat 1				Goat 2				Goat 3				Goat 4			
	Faeces		Urine		Faeces		Urine		Faeces		Urine		Faeces		Urine	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Pre-fast	1.94	1.57	0.044	0.046	1.46	1.18	0.068	—	2.96	2.08	0.176	0.024	3.37	2.49	0.182	0.019
1	1.01	1.46	—	—	1.18	1.06	—	—	3.39	2.50	—	—	4.47	3.27	—	—
2	0.86	0.62	0.024	0.402	0.45	0.36	0.072	0.244	—	—	—	—	—	—	0.202	0.274
3	0.62	0.34	—	—	0.42	0.19	—	—	—	—	—	—	—	—	—	—
4	0.57	0.32	—	—	0.66	0.24	—	—	0.93	0.70	0.348	0.337	2.10	1.35	—	—
5	—	—	0.020	1.280	—	—	0.030	1.780	—	—	—	—	0.35	0.26	—	—
6	—	—	—	—	0.47	0.10	—	—	0.44	0.28	0.072	0.324	0.42	0.29	0.088	0.308
7	0.23	0.14	—	—	—	—	—	—	0.39	0.27	—	—	0.28	0.21	—	—
8	—	—	—	—	—	—	—	—	0.64	0.44	0.018	0.507	—	—	—	—
9	0.37	0.53	0.012	0.130	0.39	0.10	0.018	1.940	0.48	0.32	—	—	0.18	0.14	0.017	0.682
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	0.30	0.23	0.008	0.489	0.18	0.14	0.024	0.506
12	—	—	—	—	—	—	—	—	—	—	—	—	0.19	0.15	—	—
Post-fast	1.92	1.36	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sheep 1																
Pre-fast	3.05	1.05	0.097	0.009	3.18	1.04	0.069	0.015	2.82	1.35	0.086	0.017	2.60	1.02	0.083	0.011
1	1.26	0.43	0.027	0.040	2.75	0.99	0.058	0.028	2.01	0.80	0.044	0.021	—	—	0.127	0.028
2	0.66	0.13	0.016	0.018	0.89	0.23	0.048	0.076	0.34	0.13	0.185	0.034	0.46	0.15	0.074	0.030
3	1.02	0.25	0.012	0.084	0.61	0.18	0.031	0.142	0.66	0.25	0.077	0.010	0.79	0.29	0.085	0.240
4	0.43	0.12	0.008	0.376	0.26	0.07	0.022	0.332	—	—	—	—	0.28	0.11	0.024	0.201
5	0.64	0.17	0.008	0.288	0.60	0.18	0.006	0.280	—	—	0.042	0.304	0.79	0.33	0.014	0.316
6	0.56	0.18	0.010	0.301	0.41	0.12	0.020	0.392	0.66	0.29	0.00	0.622	0.73	0.30	0.014	0.316
7	0.32	0.10	0.008	0.382	—	—	0.006	0.508	1.00	0.42	0.010	0.233	0.27	0.10	0.008	0.407
Sheep 2																
Pre-fast	3.05	1.05	0.097	0.009	3.18	1.04	0.069	0.015	2.82	1.35	0.086	0.017	2.60	1.02	0.083	0.011
1	1.26	0.43	0.027	0.040	2.75	0.99	0.058	0.028	2.01	0.80	0.044	0.021	—	—	0.127	0.028
2	0.66	0.13	0.016	0.018	0.89	0.23	0.048	0.076	0.34	0.13	0.185	0.034	0.46	0.15	0.074	0.030
3	1.02	0.25	0.012	0.084	0.61	0.18	0.031	0.142	0.66	0.25	0.077	0.010	0.79	0.29	0.085	0.240
4	0.43	0.12	0.008	0.376	0.26	0.07	0.022	0.332	—	—	—	—	0.28	0.11	0.024	0.201
5	0.64	0.17	0.008	0.288	0.60	0.18	0.006	0.280	—	—	0.042	0.304	0.79	0.33	0.014	0.316
6	0.56	0.18	0.010	0.301	0.41	0.12	0.020	0.392	0.66	0.29	0.00	0.622	0.73	0.30	0.014	0.316
7	0.32	0.10	0.008	0.382	—	—	0.006	0.508	1.00	0.42	0.010	0.233	0.27	0.10	0.008	0.407
Sheep 3																
Pre-fast	3.05	1.05	0.097	0.009	3.18	1.04	0.069	0.015	2.82	1.35	0.086	0.017	2.60	1.02	0.083	0.011
1	1.26	0.43	0.027	0.040	2.75	0.99	0.058	0.028	2.01	0.80	0.044	0.021	—	—	0.127	0.028
2	0.66	0.13	0.016	0.018	0.89	0.23	0.048	0.076	0.34	0.13	0.185	0.034	0.46	0.15	0.074	0.030
3	1.02	0.25	0.012	0.084	0.61	0.18	0.031	0.142	0.66	0.25	0.077	0.010	0.79	0.29	0.085	0.240
4	0.43	0.12	0.008	0.376	0.26	0.07	0.022	0.332	—	—	—	—	0.28	0.11	0.024	0.201
5	0.64	0.17	0.008	0.288	0.60	0.18	0.006	0.280	—	—	0.042	0.304	0.79	0.33	0.014	0.316
6	0.56	0.18	0.010	0.301	0.41	0.12	0.020	0.392	0.66	0.29	0.00	0.622	0.73	0.30	0.014	0.316
7	0.32	0.10	0.008	0.382	—	—	0.006	0.508	1.00	0.42	0.010	0.233	0.27	0.10	0.008	0.407
Sheep 4																
Pre-fast	3.05	1.05	0.097	0.009	3.18	1.04	0.069	0.015	2.82	1.35	0.086	0.017	2.60	1.02	0.083	0.011
1	1.26	0.43	0.027	0.040	2.75	0.99	0.058	0.028	2.01	0.80	0.044	0.021	—	—	0.127	0.028
2	0.66	0.13	0.016	0.018	0.89	0.23	0.048	0.076	0.34	0.13	0.185	0.034	0.46	0.15	0.074	0.030
3	1.02	0.25	0.012	0.084	0.61	0.18	0.031	0.142	0.66	0.25	0.077	0.010	0.79	0.29	0.085	0.240
4	0.43	0.12	0.008	0.376	0.26	0.07	0.022	0.332	—	—	—	—	0.28	0.11	0.024	0.201
5	0.64	0.17	0.008	0.288	0.60	0.18	0.006	0.280	—	—	0.042	0.304	0.79	0.33	0.014	0.316
6	0.56	0.18	0.010	0.301	0.41	0.12	0.020	0.392	0.66	0.29	0.00	0.622	0.73	0.30	0.014	0.316
7	0.32	0.10	0.008	0.382	—	—	0.006	0.508	1.00	0.42	0.010	0.233	0.27	0.10	0.008	0.407

Table IX. *Ca : P ratios in faeces of goats and sheep*

	Ca : P		
	Pre-fast	Fast	Post-fast
Goat 1	1.24	1.30	1.41
2	1.24	3.25	—
3	1.42	1.46	—
4	1.36	1.33	—
Sheep 1	2.90	3.06	—
2	3.05	2.85	—
3	2.45	2.44	—
4	2.55	2.43	—

endogenous in origin cannot be entirely ruled out. A small part probably arises from the various intestinal secretions. Also there is a marked increase in the inorganic P content of the faeces during starvation, as shown by the following figures representing mg./100 g.:

	Cow 1	Cow 2	Cow 3
Pre-fast	40	41	41
End of fast	326	139	169

The high serum P (Table VII) may necessitate the formation of a colloidal Ca-P compound, to which the kidney is impermeable, which is excreted into the gut and faeces. If, however, there is any marked catabolism of osseous tissue, the excretion of Ca and P from this source must be through the faeces, since the urinary Ca during starvation is negligible, and since it has already been shown that the urinary P can be accounted for by the muscular catabolism. Although the fact that the Ca : P ratio in faeces is roughly constant throughout is in accordance with the idea that bone is broken down and both the Ca and P are excreted by way of the faeces, this would materially alter the Ca : P ratio of the faeces, giving an increase with the goats and a decrease with the sheep, since the ratio in bone is approximately 2 : 1. Aylward & Blackwood [1936] did find a difference in the ratio of Ca : P between the pre-fast and the starvation faeces.

SUMMARY

1. A complete study has been made of the fasting catabolism of ruminants: cows, goats and sheep.
2. The daily urinary N excretion per kg. body-weight was 0.06–0.08 g. for cows, 0.12–0.20 g. for goats and 0.12–0.16 g. for sheep.
3. The daily faecal N output was 6.6 g. for cows, 0.35–0.50 g. for goats and 0.49–0.80 g. for sheep.
4. The preformed creatinine tended to diminish slightly during the fast, whereas the creatine rose markedly.
5. The purine metabolism showed a fall in the output of uric acid and allantoin but an increase in that of purine base.
6. Blood analyses showed an increase in both the protein and non-protein nitrogen fractions. Whereas the increase in protein could be explained by a slight dehydration of the blood, the increase in the N.P.N., due chiefly to an increase in the undetermined fraction, could not be explained. A marked increase in blood uric acid was noted.
7. It was also shown that even in a 12-day fast there appeared to be no catabolism of osseous tissue with ruminants.

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CL. THE DETERMINATION OF LEAD IN BIOLOGICAL MATERIALS

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FURTHER experience of the method of Tompsett & Anderson [1935] for the estimation of lead in human tissues and excreta has shown that in the analysis of different types of material modifications are an advantage in special cases. Also certain general improvements make it desirable to describe the method in more detail. A survey of the various methods for estimating minute amounts of lead in biological materials is not included as this has been done by us [1935] and more recently by Minot [1938].

The method consists essentially of three stages: (1) destruction of organic material, (2) extraction of Pb with ether as a complex with Na diethyldithiocarbamate, (3) colorimetric estimation of lead with dithizone.

Destruction of organic material

Two methods are available, viz. digestion with H_2SO_4 and some oxidant, e.g. HNO_3 or HClO_4 , and ignition.

The objection to the first method is that often large quantities of H_2SO_4 and oxidant are required and consequently large quantities of alkali are necessary for neutralization. This leads to a large blank even when the purest reagents are used.

Destruction by ignition, which avoids this high blank has been criticised on the grounds that Pb may be lost by volatilization. The writer has found that materials containing a large amount of ash, consisting in the main of phosphates, may be ignited in a silica dish over a bunsen burner without loss of Pb. When materials containing a low ash content are treated in this manner, a loss of Pb may occur. For this reason, the ash content of such materials is increased by the addition of Na phosphate. Biological materials are therefore divided into two classes:

(1) High mineral content—ignited without the addition of Na phosphate, e.g. urine, faeces, milk and bone.

(2) Low mineral content—Na phosphate added before ignition, e.g. blood, soft tissues.

The ignition method has been found to be quite satisfactory under these conditions and has been used throughout. Towards the end, ignition may be assisted by allowing the ash to cool, adding a little conc. HNO_3 and re-heating.

Separation of Pb with ether and Na diethyldithiocarbamate

Na diethyldithiocarbamate reacts with various metals to form complexes, many of which are soluble in organic solvents. Amongst the metals which occur in biological materials and react thus are Fe, Cu and Pb. Pb forms a complex which is soluble in ether. This complex is produced in acid and in alkaline solution and its formation is not inhibited by the presence of such substances as

citrate, pyrophosphate or cyanide. The formation of the Cu complex is inhibited if the solution is alkaline and contains cyanide in addition. The Fe complex is not formed if the solution is alkaline and contains in addition pyrophosphate, citrate or cyanide.

Before Pb may be estimated with the dithizone reagent it is essential to separate it from Fe and recent experience has shown that it is advantageous to separate it from Cu. Use of the above is made to achieve this. The separation is carried out by adding to a solution of the metals, Na citrate, NH_3 to make alkaline and then Na diethyldithiocarbamate. The Pb complex, which is the only one formed under these conditions, may then be extracted with ether. The presence of the Na citrate is essential so as to prevent the precipitation of hydroxides and phosphates in the alkaline medium.

This technique has been found to be quite satisfactory for urine, soft tissues and blood, but in the case of milk, bone and faeces, certain modifications have been found to be advantageous. The ash of the second class of material contains a considerable amount of Ca phosphate. As a result, sometimes in spite of the presence of citrate, troublesome precipitates of Ca phosphate appear when the mixture is made alkaline. Such precipitates may prevent the quantitative separation of Pb. For this reason, a twofold extraction is employed in the examination of these materials.

Na diethyldithiocarbamate is added to an acid solution of the ash. All the metallic complexes are formed and all are extracted with ether. The extracted metals are converted into the inorganic state and the process of extraction repeated but in an alkaline solution containing citrate and cyanide. Under these conditions, the Pb complex alone is formed, and this alone extracted by the ether.

The colorimetric estimation of Pb with dithizone

The extracted Pb is converted into the sulphate by digestion with H_2SO_4 . After digestion, water is added and then acetic acid. The mixture is then made alkaline by the addition of NH_3 . An alkaline reaction is essential for the occurrence of the reaction between Pb and dithizone and the presence of ammonium acetate ensures that the PbSO_4 is in solution.

The reaction is as follows. KCN and CCl_4 are added to the alkaline acetate solution of Pb. Dithizone solution is then added and the whole well shaken. The Pb forms a complex which dissolves in the CCl_4 to produce a red solution. The solution of dithizone in ammoniacal solutions is brown and in CCl_4 green. After the shaking process, unchanged dithizone will be distributed between the aqueous and CCl_4 phases. The next stage is to separate the CCl_4 layer and to free it from unchanged dithizone by shaking it with KCN solution, when uncombined dithizone is removed and an extract containing the red coloured Pb complex alone is obtained. This reaction is quantitative and the Pb present may be estimated by colorimetric comparison with a standard.

Certain precautions are however necessary. Excess of dithizone must be used to ensure that all the Pb present has been converted into dithizone complex, but too great an excess must be avoided.

Dithizone is very susceptible to oxidation and a substance is produced which is soluble in CCl_4 to produce a yellow coloured solution. This substance cannot be removed from CCl_4 by cyanide solutions. It is produced when Fe or Cu salts are present and by bright sunlight. In the earlier work, the influence of Fe salts was realized and they were carefully eliminated. The effect of Cu was not then known but owing to its generally low concentration in biological materials, the

effect was almost negligible. Occasionally trouble was experienced and this was found to occur in materials having a high Cu content. Upon removal of the Cu, no trouble was experienced. It has been decided, therefore, to eliminate Cu as well as Fe.

Bright sunlight causes a rapid production of the yellow oxidation product. This does not take place in ordinary diffuse light. It is believed that it is the ultraviolet component of bright sunlight which is responsible for this reaction [Tompsett, 1936]. In ordinary diffuse light the CCl_4 solution of the red coloured Pb complex will remain stable for a very long period.

Apparatus and reagents

Pyrex glassware was used throughout. Silica dishes were washed with hot dilute HCl before use. Glass-distilled water was used and filter papers were washed with dilute HCl followed by water.

The reagents used were the same as those described by Tompsett & Anderson [1935] with the following exceptions:

Dithizone reagent. Commercial dithizone contains an oxidation product which must be removed. In earlier work a mass purification was done but this has now been abandoned. A 0.1% solution of 0.1% crude dithizone in CCl_4 is prepared. Just before use, a small volume of this is shaken with an equal volume of 0.5% NH_3 . Dithizone but not the oxidation product passes into the aqueous layer. After allowing the mixture to settle, the ammoniacal extract is separated and used directly.

Na_2HPO_4 —10% in water—lead-free. Before use the requisite volume of a stock solution is introduced into a separating funnel, sodium diethyldithiocarbamate added and the mixture shaken vigorously with ether. After settling, the lead-free aqueous layer is run off.

The method

In the following, details of the method as applied to various materials are described. The final colorimetric estimation is the same in each case.

A. Extraction of lead

Urine. 500 ml. of urine are evaporated to dryness in a silica dish in a hot air oven. The residue is then ashed by ignition over a bunsen burner in a fume cupboard.

The ash is dissolved in 100 ml. of water containing 5 ml. of conc. HCl. The solution is transferred to a separating funnel, 50 ml. of 20% Na citrate added and the mixture made alkaline by the addition of ammonia. 5 ml. of 10% KCN are added and the mixture cooled thoroughly. 5 ml. of 2% Na diethyldithiocarbamate are added and the mixture extracted 3 times with ether, 25 ml. on each occasion. The ether extracts which are separately washed with water are transferred to a hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. conc. H_2SO_4 and 1 ml. HClO_4 to destroy organic matter.

The residue is diluted with water, 1 ml. glacial acetic acid added, followed by 5 ml. ammonia sp.gr. 0.88, and the mixture diluted to 25 ml. by the addition of water.

Soft tissues. To 100 ml. of lead-free 10% Na_2HPO_4 in a silica basin are added 100 g. fresh tissue. After drying in a hot air oven, the material is ashed.

The ash is dissolved in 100 ml. water containing 10 ml. conc. HCl. The procedure is then as for urine.

Blood. To 100 ml. of lead-free 10 % Na_2HPO_4 in a silica dish are added 20 ml. blood. After drying in a hot air oven, the material is ashed.

The ash is dissolved in about 50 ml. water containing 5 ml. conc. HCl . After transference to a separating funnel, it is cooled, 5 ml. of 20 % Na citrate added and the mixture made alkaline to litmus by the addition of ammonia. 5 ml. of 10 % KCN are added, followed by 2 ml. of 2 % Na diethyldithiocarbamate. The mixture is extracted twice with ether, 20 ml. being used on each occasion. The ether extracts, which on each occasion are washed with water, are transferred to a 100 ml. hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 0.2 ml. conc. H_2SO_4 and 0.5 ml. HClO_4 . To the digest are added 3.5 ml. water, 0.2 ml. glacial acetic acid and 1.5 ml. ammonia, sp.gr. 0.88.

Faeces. 10 g. of dried faeces are ashed in a silica basin. The ash is dissolved in 100 ml. water containing 10 ml. conc. HCl . The solution is then made up to a volume of 200 ml. by the addition of water.

50 ml. of the ash solution are introduced into a separating funnel and 10 ml. of 2 % Na diethyldithiocarbamate added. The mixture is extracted 3 times with ether, 25 ml. on each occasion. The ether extracts are collected in a hard glass flask and the ether evaporated off. The residue is digested with 1 ml. conc. H_2SO_4 and 1 ml. HClO_4 .

The residue is diluted with water, 1 ml. conc. HCl added and the mixture heated to dissolve the digest. The solution is transferred to a separating funnel to make a volume of about 50 ml., 5 ml. of 20 % Na citrate are added and the mixture made alkaline to litmus by the addition of NH_3 . 5 ml. of 10 % KCN are added, followed by 5 ml. of 2 % Na diethyldithiocarbamate. The mixture is then extracted 3 times with ether, 25 ml. being used on each occasion. The combined ether extracts are transferred to a hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. conc. sulphuric acid and 1 ml. perchloric acid. To the residue are added, water, 1 ml. glacial acetic acid and 5 ml. ammonia, sp.gr. 0.88, and the mixture is diluted to 25 ml. with water.

Bone. 20 g. of bone are ashed in a silica dish. The ash is dissolved in water containing HCl and the solution diluted to 200 ml., 50 ml. of this are taken and treated as in the case of faeces.

Milk. 500 ml. of milk are evaporated to dryness in a silica dish in a hot air oven and then ashed. The ash is dissolved in water containing HCl and treated as in the case of faeces.

B. The colorimetric estimation of lead

1. *Preparation of standard.* The following mixture is prepared. To 1 ml. conc. H_2SO_4 are added a little water, 1 ml. glacial acetic acid and 5 ml. ammonia, sp.gr. 0.88 and the whole diluted with water to a volume of 25 ml. giving ammonium acetate.

A known amount of Pb is added to 5 ml. of this mixture. To this are added 5 ml. of 1 % KCN and 10 ml. of CCl_4 . To this mixture is added drop by drop an ammoniacal solution of dithizone reagent, with constant shaking until excess has been added. Too great an excess must be avoided. Sufficient excess is indicated when the CCl_4 layer has reached its maximum intensity of redness and the aqueous layer is tinged brown. At this stage, the aqueous layer is removed and discarded. The CCl_4 layer, containing the red coloured lead complex, is shaken repeatedly with aliquots of 5 ml. of 1 % KCN until the aqueous layers

are no longer coloured. The CCl_4 layer may be passed through a filter to remove droplets of water and is then ready for comparison.

A range of standards may be prepared but the writer prefers to use a standard containing 0.02 mg. Pb, and to take the requisite amount of the unknown to conform to this. This standard may be obtained by using 2 ml. of a standard solution of lead acetate, containing 0.01 mg. Pb per ml.

2. *Preparation of unknown urine, faeces, soft tissues, bone and milk.* The extracted Pb is contained as PbSO_4 in a solution of ammoniacal ammonium acetate having a volume of 25 ml. For the estimation, 5 ml. of the solution are taken and to this are added 5 ml. of 1% KCN and 10 ml. of CCl_4 . The colour is developed as described above.

In the event of the Pb content of the unknown being low, 10 ml. of the solution are used, the amounts of the other reagents being the same. If the Pb content of the unknown is high, a smaller volume than 5 ml. is used. In this case the solution is diluted to 5 ml. by the addition of an ammoniacal ammonium

Table I. *The recovery of added Pb*

	Initial Pb content mg.	Pb added mg.	Total Pb found mg.	Pb recovered mg.
Urine (500 ml.)	0.120	0.050	0.163	0.043
	0.120	0.100	0.215	0.095
	0.120	0.200	0.308	0.188
	0.120	0.250	0.362	0.242
	0.120	0.500	0.625	0.505
	0.042	0.020	0.079	0.037
	0.042	0.050	0.096	0.054
	0.042	0.100	0.140	0.098
	0.155	0.200	0.360	0.205
	0.155	0.400	0.565	0.410
Faeces (10 g.)	0.030	0.200	0.235	0.205
	0.030	0.500	0.549	0.519
	0.030	1.000	1.036	1.006
	0.042	0.030	0.079	0.037
	0.042	0.050	0.096	0.054
	0.042	0.100	0.140	0.098
	0.031	0.040	0.072	0.041
	0.031	0.080	0.115	0.084
	0.031	0.120	0.154	0.123
Milk (500 ml.)	0.050	0.050	0.105	0.055
	0.050	0.100	0.145	0.095
	0.050	0.200	0.248	0.198
	0.050	0.500	0.560	0.510
Liver (100 g. fresh)	0.243	0.050	0.299	0.056
	0.243	0.100	0.350	0.107
	0.243	0.200	0.440	0.197
	0.243	0.400	0.640	0.397
Blood (20 ml.)	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
	16	10	27	11
	16	20	37	21
	16	40	58	42
	19	10	28	9
	19	20	39	20
	19	40	58	39
	12	10	22	10
	12	20	34	22
	12	40	53	41

acetate solution, having the same composition as that used to prepare the standard. The amounts of the other reagents are the same. The development of the colour is best carried out in glass-stoppered tubes.

Blood. In the case of blood, the whole of the Pb-containing solution is used. To the mixture containing the Pb in the flask used for the digestion, add 5 ml. 1% KCN and 10 ml. CCl_4 . The colour is developed as above.

C. Blank

A blank should always be done on a new set of reagents. In estimating the blank, the complete process is carried out. The blank is small and is thus difficult to estimate accurately so that the following method has been adopted. Before colorimetric estimation, 0.02 mg. Pb is added to the blank. This, after the development of the colour with dithizone, is compared with a standard containing 0.02 mg. Pb. The blank is then calculated from the difference.

A series of recovery experiments is shown in Table I.

DISCUSSION

It will be seen from the Table that added Pb could be estimated accurately. The method is fairly quickly performed. It is designed so that little apparatus is required and so that large scale purification of reagents is avoided.

The accurate estimation of Pb is of some importance as Pb is a "normal" hazard which at times may become unduly large, and it is also an important industrial hazard. In a recent publication Tompsett & Anderson [1939] have shown the value of Pb estimations, especially in the blood, in the diagnosis of plumbism.

SUMMARY

The estimation of lead in biological materials is described. The method comprises an earlier one in which are incorporated certain modifications.

In conclusion I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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CLI. THE INFLUENCE OF CERTAIN CONSTITUENTS OF THE DIET UPON THE ABSORPTION OF LEAD FROM THE ALIMENTARY TRACT

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LEAD may be absorbed from the respiratory tract, from the alimentary tract and from the skin. In industrial Pb poisoning, absorption from the respiratory tract is usually the important factor. In the "normal" hazard, absorption is chiefly from the alimentary tract. This at times may be largely due to contamination of foodstuffs and drinking water with undue amounts of Pb. The object of the experiments to be described was to see whether variation in the composition of the diet had any influence upon the absorption of Pb from the alimentary tract.

Aub *et al.* [1926] compared the degrees of absorption of Pb in two groups of cats, one group being on a milk diet and the other on a milk-free diet. They could detect no marked difference in the amounts of Pb absorbed by the two groups and concluded that the amounts of Ca in the diet had no influence upon the amount of lead absorbed from the alimentary tract. Sobel *et al.* [1938], in a study of experimental Pb poisoning in rats, found that about twice as much Pb was absorbed on a diet containing viosterol as without it.

EXPERIMENTAL

Adult male mice were the animals used throughout this work. Two basic diets were used, namely a low Ca diet and a high Ca diet, of the following compositions.

<i>Low Ca diet</i>		<i>High Ca diet</i>	
Shelling [1932]			
	g.		g.
Whole wheat flour	400	Whole wheat flour	700
Casein	100	Whole milk powder	300
Corn starch	325	Marmite	50
Wheat gluten	50		
Olive oil	40		
NaCl	20		
KCl	15		
Butter	Omitted		

In the first series of experiments, the effects of high and low Ca diets upon the absorption of Pb were studied.

The mice were fed with the specified diets (2.5 g. per mouse per day), to which were added supplements of Pb in the form of lead acetate solution. Each mouse was housed in a separate glass jar, which was floored with sawdust. No restriction upon the amount of water drunk was made. The amount of diet supplied was constant per mouse per day. The diet together with the Pb supplements was made up into a thick paste with water and supplied in a porcelain dish, thus minimizing loss due to spillage. No difficulty was experienced in obtaining complete consumption of the daily diets by the mice. The experimental period was 14 days. At the end of this period, the jars were cleaned out and the animals placed upon the high Ca diet for 4 days to remove unabsorbed Pb from their alimentary tracts. The animals were then killed and the Pb content of the whole animal determined by the method published previously [Tompsett & Anderson, 1935; Tompsett, 1939]. The Pb content of control animals and also that of unsupplemented diets were determined.

It was found that absorption of Pb was not very marked with the high Ca diet but was high upon the low Ca diet. In the latter case, absorption increased with increase in the amount of added Pb.

Table I. *Experimental period, 14 days*

The results are expressed as (A) mg. total Pb, (B) mg. Pb per 100 g. body wt.

Supplement of Pb
(mg. Pb per mouse
per day)

	0.05		0.10		0.50		1.00	
	A	B	A	B	A	B	A	B
Low Ca diet								
Exp. 1	0.046	0.224	0.159	0.795	0.254	1.270	0.445	2.42
2	0.143	0.681	0.211	1.055	0.510	2.55	0.909	5.05
3	0.057	0.259	0.149	0.709	0.200	0.87	0.385	1.68
4	0.133	0.665	0.182	0.867	0.450	2.37	0.666	1.33
Average		0.457		0.856		1.76		2.63
High Ca diet								
Exp. 5	0.033	0.173	0.042	0.210	0.055	0.282	0.057	0.317
6	0.042	0.221	0.054	0.284	0.042	0.200	0.050	0.217
Average		0.197		0.247		0.241		0.267
Low Ca diet + Ca glycerophosphate (high Ca diet)								
Exp. 7	0.027	0.142	0.028	0.155	0.029	0.129	0.028	0.143
8	0.026	0.108	0.027	0.117	0.030	0.115	0.036	0.180
Average		0.125		0.136		0.122		0.162
Low Ca diet + olive oil								
Exp. 9	0.133	0.633	0.182	0.860	0.250	1.25	0.366	1.74
10	0.125	0.571	0.252	1.200	0.714	3.10	0.833	3.97
Average		0.602		1.030		2.18		2.86
Low Ca diet + cod liver oil								
Exp. 11	0.128	0.609	0.222	0.925	0.714	3.10	0.800	4.21
12	0.133	0.665	0.159	0.757	0.360	2.00	0.500	2.63
Average		0.637		0.841		2.55		3.42
High Ca diet + cod liver oil								
Exp. 13	0.029	0.132	0.040	0.167	0.046	0.230	0.054	0.135

Pb content of control animals = 0.020 ± 0.003 mg. Pb.

Pb content of the high Ca diet = 0.002 mg. Pb per mouse per day.

Pb content of the low Ca diet = 0.002 mg. Pb per mouse per day.

In view of this, experiments were then carried out in which mice were fed with low Ca diet containing added supplements of Pb, to which had been added Ca glycerophosphate (0.5 g. per mouse per day). This in effect converted the low Ca diet into a high Ca diet. Here again the effect of the high Ca diet was to produce only a small absorption of Pb.

The effects of fat and vitamin D upon the absorption of Pb were then studied. It was considered that fat might hinder the absorption of Pb by the formation of insoluble Pb soaps. In this series of experiments, the mice were placed upon the low Ca diet, containing supplements of added Pb to which was added olive oil at the rate of 1 ml. per mouse per day. No specific influence of fat upon the absorption of Pb could be detected. It was considered that vitamin D might aid the absorption of Pb in the same way as it assists the absorption of Ca. In this series of experiments the mice were placed on (a) the high Ca diet, (b) the low Ca diet containing supplements of added Pb, to which was added cod liver oil at the rate of 3 drops per mouse per day. The experimental results obtained did not indicate any marked influence by vitamin D upon the absorption of Pb.

The results obtained are shown in Table I.

DISCUSSION

Examination of the influence of variation in the concentration of certain constituents of the diet upon the absorption of Pb from the alimentary tract in mice has shown that from a high Ca diet the absorption of Pb was small and was not influenced to any great extent by the amount of Pb administered. Upon a low Ca diet, the amount of Pb absorbed was large and depended to a large extent upon the amount of Pb administered.

It is interesting to speculate as to the reasons for this. According to Shields *et al.* [1938], absorption does not take place from the stomach but takes place rapidly from the small intestine. The phosphate content of both diets is high and as a result the Pb will be chiefly present as Pb phosphate. The solubility of this Pb phosphate in the intestinal contents, and hence its capacity to be absorbed, will depend to a great extent upon the reaction of the intestinal contents. It is suggested that upon the low Ca diet, the reaction of the intestinal contents tend to become more acid whereas on the high Ca diet the contents tend to become more alkaline.

Although large amounts of Pb were absorbed on a low Ca diet, the absorption by animals receiving the same quantities of Pb in different experiments showed marked variation. Other important factors must be involved, e.g. the speed with which contents pass through the intestine, the amount of water consumed, the capacity to excrete absorbed Pb etc.

No specific action of vitamin D upon the absorption of Pb could be observed. Upon a high Ca diet the absorption of Pb was low and upon a low Ca diet the absorption of Pb was of the same order as that on a diet which did not contain additional vitamin D.

Large quantities of fat in the diet did not appear to influence the absorption of Pb.

SUMMARY

The effect of variation in the concentration of certain constituents of the diet upon the absorption of Pb from the alimentary tract of mice has been studied.

On high Ca diets, the absorption of Pb was low while upon low Ca diets it was high. Fat and vitamin D were without influence.

I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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CLII. FACTORS INFLUENCING THE FORMATION OF ROBISON ESTER

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COMPOUNDS which influence Robison ester formation from glycogen and inorganic phosphate in muscle extract may act either on the first step: glycogen + inorganic phosphate \rightleftharpoons glucose-1-phosphate (Cori ester)—or on the second step: Cori ester \rightarrow hexose-6-phosphate (Robison ester), or on both these steps. Several factors which increase or inhibit Robison ester formation could be shown to act on one or the other of the two processes. Glucose inhibits the initial phosphorylation [Lehmann, 1938, 1; Cori *et al.* 1939]. Insulin influences the second step [Lehmann, 1938, 2, 3]. Hexosediphosphate activates the formation of Cori ester [Gill & Lehmann, 1939]. KCN and reduced glutathione increase Robison ester formation [Gill & Lehmann, 1939]. Cori *et al.* [1939] report that they could find no influence of KCN and reduced glutathione on Cori ester formation, which indicates that these factors act upon the second step, the conversion of Cori ester into Robison ester.

In this paper experiments are reported which were performed to elucidate the exact point of attack of various factors. The methods used are identical with those given in a previous communication in greater detail [Gill & Lehmann, 1939]. The experiments were performed at 37°. When adenylic acid and Mg were added this is specially mentioned. The first was used as the Na salt and the second as the chloride. Old extracts were used and usually no coenzyme was added. It was found that under these conditions an appreciable amount of Cori ester was formed, the accumulation or disappearance of which it was possible to study. All values are expressed as mg. per ml. reaction mixture.

Glutathione

More Robison ester is formed in the presence of SH glutathione than in its absence. In cases where the total hexosemonophosphate production remains

Table I. *The influence of glutathione on Cori ester and Robison ester formation*

Time min.	Addition	Cori ester formed mg.	Robison ester formed mg.
5 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 2. Initial glycogen 6 mg. Initial P 1.3 mg.			
60	—	0	1.1
120	—	0.1	2.1
120	At 60 min. + M/20 GSSG	0.7	1.3
15 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 4. Initial glycogen 10 mg. Initial P 1.1 mg.			
45	—	0.5	2.0
	M/20 GSH	0.2	2.2
	M/100 GSH	0.3	2.3
	M/40 GSSG	0.9	0.6
	M/200 GSSG	0.8	1.2

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unaltered, the increase in Robison ester is due to a loss in Cori ester (Table I, Exp. 2). This supports the view that reduced glutathione acts only on the step Cori ester \rightarrow Robison ester. Strongly oxidizing compounds like quinone and oxidized catechol effect a complete inhibition of phosphorylation. When the mildly oxidizing agent SS glutathione is used a difference can be observed in the two steps of Robison ester formation regarding their sensitivity towards oxidation. The initial phosphorylation takes place whereas the conversion of the 1-ester into the hexose-6-phosphate is inhibited (Table I).

Alloxan and caffeine

Alloxan is an oxidizing agent [Strecker, 1862; Wieland & Bergel, 1924]. Its effect on thiol groups of proteins [see Labes & Freisburger, 1930] has been employed for inactivation of enzymes by oxidizing their SH groups [Purr, 1935, 1, 2; Hopkins *et al.* 1938; Rapkine, 1939]. Alloxan inhibits Robison ester formation (Table III). Whilst SS glutathione even at very high concentrations only inhibits the step Cori ester \rightarrow Robison ester, alloxan at concentrations above $M/100$ inhibits both Cori and Robison ester formation (Table III). Experiments were made to compare its action with that of caffeine, which at a concentration of $M/100$ was found to have an inhibitory influence on both Robison ester and Cori ester formation (Table II). At lower concentrations of the purine derivative only Robison ester formation is inhibited (see also Tables III and IV). Yet whereas SS glutathione allows accumulation of Cori ester to go on whilst Robison ester formation is inhibited, the lower concentrations of alloxan and caffeine are still able to prevent this increase (Tables I and III).

Table II. *Influence of caffeine on Cori ester and Robison ester formation*

10 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 4. Initial glycogen 6 mg. Initial P 1.6 mg. Time of exp. 30 min.

Addition	—		+ 0.15 mg. Mg		+ 1.5 mg. adenylic acid	
	Cori ester formed	Robison ester formed	Cori ester formed	Robison ester formed	Cori ester formed	Robison ester formed
—	0.9	2.3	0.8	2.9	1.2	2.7
$M/100$ Caffeine	0.3	1.1	0.5	0.9	0.6	2.0

Table III. *Influence of alloxan and caffeine on Cori ester and Robison ester formation*

15 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 4. Initial glycogen 10 mg. Initial P 1.1 mg. Time of exp. 45 min.

Additions	Cori ester formed	Robison ester formed
—	0.5	2.0
$M/20$ Alloxan	0.3	0.2
$M/100$ Alloxan	0.5	0.9
$M/500$ Alloxan	0.5	1.3
$M/100$ Caffeine	0.2	0.9
$M/500$ Caffeine	0.5	1.4

The action of alloxan also differs from that of SS glutathione in that it is not completely reversed by SH glutathione. In Table IV is shown the annihilation of the effect of SS glutathione by an equivalent amount of the reduced tripeptide; $M/25$ reduced glutathione only partially reverses the action of $M/125$ alloxan

and has no influence on the effect of *M*/150 caffeine (Table IV). The action of caffeine on protein in general has usually been described as denaturation. There are indications that it exercises a dehydrating influence [Handovsky, 1910; Pauli & Falek, 1912; see also Szelőczy, 1929 and Brühl, 1929]. The last-named author finds evidence for a complex formation between amino-acids and caffeine. Alloxan may act in a similar way besides oxidizing the thiol groups.

Table IV. *Different reversibility by SH glutathione of the action of SS glutathione, alloxan and caffeine*

16 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 5. Initial glycogen 8 mg. Initial P 0.9 mg. Time of exp. 60 min.

Additions	Cori ester formed	Robison ester formed
---	0.6	1.9
<i>M</i> /25 GSH	0.3	2.4
<i>M</i> /50 GSSG	1.0	0.5
<i>M</i> /50 GSSG + <i>M</i> /25 GSH	0.6	2.0
<i>M</i> /125 Alloxan	0.6	0.8
<i>M</i> /125 Alloxan + <i>M</i> /25 GSH	0.7	1.4
<i>M</i> /150 Caffeine	0.7	0.7
<i>M</i> /150 Caffeine + <i>M</i> /25 GSH	0.7	0.7

Zinc and cobalt

Zn has an inhibiting, Co an increasing effect on Robison ester formation. Zn in small concentrations only inhibits the Robison ester formation and not the Cori ester formation. Co increases Robison ester formation but has no influence on the production of Cori ester (Table V).

Table V. *Influence of Zn and Co on Cori ester and Robison ester formation*

26 days old undialysed rabbit muscle extract. Extract : total volume = 1 : 3.4. Initial glycogen 5.9 mg. Initial P 1.4 mg. Time of exp. 80 min.

Additions	Cori ester formed	Robison ester formed
---	0.1	2.6
<i>M</i> /850 ZnSO ₄	1.0	1.6
<i>M</i> /850 Co(NO ₃) ₂	0.1	3.6

It has to be emphasized that the effect of Co cannot be exactly the same as that of the reducing compounds. It acts at a much lower molarity and actually removes free SH groups. This was demonstrated in a model experiment with thiolacetic acid [Gill & Lehmann, 1939], and can also be seen by incubating a washed muscle brei in a *M*/200–*M*/500 Co solution for a few minutes. The absence of free SH groups can be demonstrated by the negative nitroprusside test after washing. Zn does not have this effect on muscle protein and had no effect on thiolacetic acid.

Experiments on Cori ester

The increasing or decreasing influences of several compounds on Robison ester formation can be seen also by studying the conversion of Cori ester into Robison ester. I am grateful to Dr C. S. Hanes for a generous supply of the ester which he is able to prepare on a large scale by means of a new method which is not yet published.

Conversion of Cori ester into Robison ester

10 days old undialysed rabbit muscle extract + 1.9 mg. Cori ester. Extract : total volume = 1 : 4.
Time of exp. 8 min.

Additions	Robison ester formed	± % of Robison ester formed without addition of agents
—	0.3	—
M/20 GSSG	0.1	- 70 %
M/10 GSH	0.6	+ 100 %
M/5 KCN	1.2	+ 300 %
M/100 Caffeine	0.1	- 70 %
M/1500 Zn	0.1	- 70 %
M/1500 Co	0.8	+ 170 %
1 % Glucose	0.3	± 0

It may be noted that glucose as already reported [Gill & Lehmann, 1939] does not influence the step Cori ester → Robison ester.

SUMMARY

SH glutathione increases Robison ester formation but has no influence on Cori ester formation, SS glutathione inhibits Robison ester formation but has no influence on Cori ester formation.

Alloxan and caffeine both inhibit Robison ester formation, they also inhibit Cori ester formation at concentrations higher than about M/100.

The inhibition by SS glutathione can be completely reversed by SH glutathione. SH glutathione partially reverses the alloxan inhibition, it has no influence on the caffeine inhibition.

Cobalt acts on Robison ester formation like reduced glutathione, yet it is effective at much lower concentrations and it removes SH groups from washed muscle brei.

Zinc in small concentrations inhibits the formation of Robison ester but not the formation of Cori ester. Although it acts in this respect like oxidized glutathione, it is effective at much lower concentrations and it does not remove SH groups from washed muscle brei.

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CLIII. *L*-MALIC DEHYDROGENASE AND CODEHYDROGENASE OF *BACTERIUM COLI*

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QUASTEL & WHETHAM [1924] stated that resting suspensions of *Bact. coli* will not reduce methylene blue in the presence of malate. However, den Dooren de Jong [1926] showed that a strain of *Bact. coli* can grow in an inorganic medium containing NH_3 with malic acid as the sole source of carbon. Booth & Green [1938] found a malic dehydrogenase in the cell-free juice obtained by crushing *Bact. coli* in the bacterial mill. The following paper establishes the existence of the enzyme in the organism, deals with its formation, extraction from the cell, properties, coenzyme requirements and with the identification of the oxidation product.

Methods

The organism used was the stock strain of *Bact. coli* in use in this laboratory. Washed suspensions of the organism were prepared as described in previous papers. The enzyme activity was investigated first by the methylene blue technique. The following quantities were used in Thunberg tubes: 1 ml. phosphate buffer at pH 7.2; 0.2 ml. 0.5% methylene blue; 1 ml. *M* 10 *L*-malate (water in controls); 0.2 ml. 2 *M* NaCN adjusted to pH 8; 1 ml. washed suspension of enzyme preparation and 0.5 ml. water, coenzyme solution or other addition as described below. The tubes were evacuated, equilibrated in a bath at 37°, the substrate tipped in from the hollow stopper and the time measured for the dye to be completely decolorized.

Where O_2 uptakes were studied, the experiments were carried out in Warburg manometers under conditions which are set out in detail later.

Demonstration of the enzyme in washed suspensions of *Bact. coli*

This enzyme is difficult to demonstrate in the intact organism owing to its small amount or low activity. Its presence is best shown in the presence of

Table 1. *Demonstration of malic dehydrogenase in Bact. coli*

	Additions (ml.) to tubes							
	1	2	3	4	5	6	7	8
(a) <i>M</i> /25 buffer pH 7.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
(b) 0.5% methylene blue	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
(c) <i>M</i> /10 Na malate	1.0	—	1.0	—	1.0	—	1.0	—
(d) 2 <i>M</i> NaCN	0.2	0.2	0.2	0.2	—	—	—	—
(e) Cozymase 2 mg./ml.	0.5	0.5	—	—	0.5	0.5	—	—
(f) Water	—	1.0	0.5	1.5	0.2	1.2	0.7	1.7
(g) Bacterial suspension 9 mg. dry wt./ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Reduction time (min.)	32	∞	54	∞	8	11	40	58

Additions (a)–(d) in quantities as in tube 1 remain the same in subsequent experiments.

NaCN and with the addition of coenzyme I (see later). The cyanide inhibits the high blank due to the large quantity of organism necessary and also acts by fixing the keto-group of the oxaloacetic acid formed [Green, 1936; Green & Williamson, 1937]. An experiment demonstrating the action of these additions is set out in Table I.

Extraction of the enzyme. For this purpose the wet-crushing mill of Booth & Green [1938] was used. The organisms obtained from the surface of broth-agar in 30 Roux bottles were washed off, centrifuged and washed twice, made up to 40 ml. (total dry weight of organism = about 3 g.) and circulated in the mill for 2½ hr. The crushed material was centrifuged for 30 min. at 3000 r.p.m. This gives an opaque juice *A* (25–30 mg./ml.) and a sediment *B*. *A* was further centrifuged for 20 min. at 11,000 r.p.m., giving a clear brown fluid *A*₁ (18–20 mg./ml.). *A*₁ was rendered water-clear by passage through a Seitz filter to give *A*₂ (3–4 mg./ml.).

Table II. *Malic dehydrogenase activity: washed suspension of Bact. coli*

	Additions (ml.) to tubes					
	1	2	3	4	5	6
(a) <i>M</i> /25 buffer pH 7.2	1.0	1.0	1.0	1.0	1.0	1.0
(b) 0.5% methylene blue	0.2	0.2	0.2	0.2	0.2	0.2
(c) <i>M</i> /10 Na malate	1.0	1.0	—	1.0	1.0	—
(d) 2 <i>M</i> NaCN	0.2	0.2	0.2	0.2	0.2	0.2
(e) Cozymase 2 mg./ml.	—	—	—	0.5	0.5	0.5
(f) Water	0.5	0.5	1.5	—	—	1.0
(g) Bacterial suspension 7.2 mg./ml.	1.0	1.0	1.0	1.0	1.0	1.0
Reduction time (min.)	68	59	>180	24½	23½	>180
<i>Q</i> _{MB}	3.7	4.2	<1	10.2	10.6	<1

Table III. *Distribution of malic dehydrogenase in crushed material*

Source of enzyme	Activity with cozymase added	Activity without cozymase
Washed suspension of organism	100	27
Crushed material	122	2
Washed sediment <i>B</i>	65	< 1
Juice <i>A</i>	185	< 1
Juice <i>A</i> ₁	189	< 1
Juice <i>A</i> ₂	190	< 1

The sediment *B* was washed twice in distilled water on the centrifuge. The dry weight of all fractions was determined by drying to constant weight in a steam oven and the *Q*_{MB} as malic dehydrogenase determined, where *Q*_{MB} = μl. O₂ equivalent to the methylene blue reduced/hr./mg. dry weight of preparation. Table II shows the determination in full for the washed suspension of intact organism and Table III shows the activities of the various fractions expressed as % of that of the original washed suspension of organism (=100) in the presence of cozymase and NaCN.

The activity of the malic dehydrogenase is found to reside mainly in the juice, the sediment after washing being markedly less active than the original suspension while the final water-clear juice *A*₂ has an activity per unit dry weight of approximately twice that of the organism.

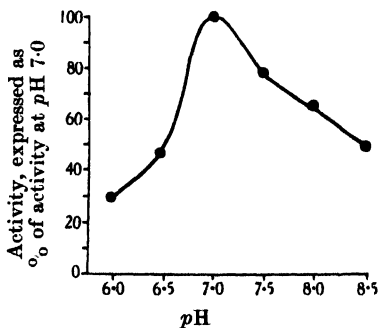


Fig. 1. Effect of pH on malic dehydrogenase activity (juice *A*).

Fig. 1 shows the effect of *pH* on the activity of the malic dehydrogenase of juice *A* in the presence of cyanide and a constant excess of cozymase.

Nature of the coenzyme. The results in Table III show that a coenzyme is essential for the action of malic dehydrogenase with methylene blue. This coenzyme is present in the cozymase preparation from yeast [Green & Brosteaux, 1936] but this preparation contains considerable impurities. Accordingly the additions listed in Table IV were tested for their codehydrogenase activity in the place of the cozymase. Also the cozymase solution was boiled at *pH* 12 and then readjusted to *pH* 7 before testing, this treatment inactivating the coenzymes I and II [Green & Brosteaux, 1936]. From Table IV it appears that boiled

Table IV. *Nature of codehydrogenase*

Tubes were made up as usual with regard to additions (a)–(d). 1 ml. of juice *A*₁ was used as a source of malic dehydrogenase (g). 0.5 ml. of the following additions was used as source of codehydrogenase.

Source of codehydrogenase	Reduction time
Water	> 4 hr.
Cozymase 2 mg./ml.	12 min.
Cozymase previously boiled at <i>pH</i> 12	> 4 hr.
Boiled organism 10 mg./ml.	39 min.
(Control on organism = > 4 hr.)	
Boiled organism 40 mg./ml.	18 min.
(Control on organism = > 4 hr.)	
<i>M</i> /600 nicotinamide	> 4 hr.
<i>M</i> /600 adenylic acid	> 4 hr.
<i>M</i> /600 ribose	> 4 hr.
<i>M</i> /600 (nicotinamide + ribose + adenylic acid)	> 4 hr.

bacteria will act as a source of the codehydrogenase as also will the untreated cozymase solution, but not after treatment. The various decomposition products of coenzyme I are inactive so that the organism is unable to synthesize the coenzyme from these substances with sufficient rapidity to be active in these experiments.

In order to determine whether the active substance in boiled bacteria—which must presumably be the natural codehydrogenase—is identical with coenzyme I, the following experiments were carried out.

A thick washed suspension of the bacteria (20 mg./ml.) was boiled in distilled water for 5 min., cooled and the supernatant fluid spun off. The sediment of boiled cells was washed once and then resuspended in the original volume of water. The coenzyme activities of the sediment, supernatant and whole boiled suspension were compared with that of cozymase. From the results in Table V it is seen that the entire activity of the boiled suspension of bacteria resides in

Table V. *Distribution of codehydrogenase in boiled suspension of bacteria*

Tubes contained the standard quantities of additions (a)–(d). 1 ml. of juice *A*₁ was used as a source of malic dehydrogenase (g). 0.5 ml. of the following additions was used as source of coenzyme.

Source of coenzyme	Reduction time
1. Water control	> 4 hr.
2. Cozymase 2 mg./ml.	9½ min.
3. Boiled organism 40 mg./ml.	19 min.
(Control on organism > 4 hr.)	
4. Supernatant fluid from (3)	18 min.
5. Sedimented cells from (3)	> 4 hr.

the extracted supernatant fluid and this can therefore be used as a source of the codehydrogenase.

It has been shown that the malic dehydrogenase prepared from pig's heart-muscle requires coenzyme I specifically [Green, 1936]. We have shown that the supernatant fluid from boiled *Bact. coli* will also act as coenzyme for this system and the following data show that the ratio of codehydrogenase activity of coenzyme I/supernatant is constant for the pig's heart enzyme and the enzyme in the bacterial juice preparation (Fig. 2).

Curve 1 shows the relation between the reduction time, under the standard conditions set out above, and quantities of coenzyme I added for the malic dehydrogenase preparation from pig's heart. Curves 2 and 3 show the same relation for two preparations of malic dehydrogenase from *coli*-juice (fraction A, Table III). In each case the reduction time was also determined in the absence of cozymase but with the addition of 1, 0.5 and 0.20 ml. of supernatant fluid obtained from a boiled suspension of bacteria (20 mg./ml.) by centrifuging off the whole cells and then passing the centrifugate through a Seitz filter. It is seen that not only does 1 ml. of supernatant correspond to the same amount of coenzyme I whether tested against the heart enzyme or the *coli* enzyme but also that this remains true on dilution. Further, halving the quantity of supernatant added is roughly equivalent to halving the quantity of coenzyme I. As coenzyme I is the only substance in the cozymase preparation which acts as codehydrogenase for the malic dehydrogenase preparation from heart [Green, 1936], it follows that the substance in the supernatant which is active as codehydrogenase for the malic dehydrogenase from *coli* must also be coenzyme I.

Reduction of coenzyme I by the extracted enzyme. The reduction of coenzyme I by the dehydrogenase extracted from the organism in juice A can be demonstrated as follows: 15 mg. cozymase preparation (15% coenzyme I) were dissolved in 2 ml. water and solid Na_2HPO_4 then added until $\text{pH}=8$. Then 0.4 ml. M NaCN (neutralized to $\text{pH } 8$) and 1 ml. M Na malate were added. 2 ml. of juice A were then added and the whole incubated *in vacuo* at 37° for 30 min. At the end of that time, the reaction mixture was brought to the boil and the protein filtered off. The filtrate was diluted 1 in 5 and a sample examined in the Hilger Spekker spectrophotometer. The spectrum of reduced coenzyme I was obtained with a band at $345 \text{ m}\mu$, $\log I_0/I=1.0$. A similar experiment carried out with boiled enzyme gave $\log I_0/I=0.2$ at $345 \text{ m}\mu$.

Coenzyme factor. The juice A reduces methylene blue in the presence of *l*-malate and coenzyme I. In order to determine whether coenzyme factor [Dewan & Green, 1937; 1938] is a component of this system, the enzyme was purified in such a way that the factor would be destroyed. This was carried out as follows [Green & Dewan, 1938]: the juice was mixed at 0° with 3 vol. cold acetone. The precipitate was filtered off and washed with acetone and ether.

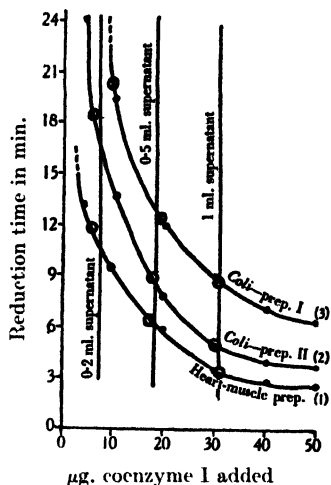


Fig. 2. (a) Effect of addition of cozymase on reduction time obtained with malic dehydrogenase preparations from *coli*-juice and heart muscle $\bullet-\bullet-$. (b) Effect of addition of supernatant fluid from boiled organism on reduction time with the same preparations $\circ-\circ-$. Demonstrates that active substance in the supernatant fluid is coenzyme I (see text).

The dried powder was rubbed up with the original volume of water and the suspension dialysed overnight at 0°. The precipitate was centrifuged and discarded. The solution was then treated for 10 min. at 52°.

The Q_{MB} of the preparation with the usual quantities of malate, coenzyme I and cyanide was 13. On the addition of 0.1 ml. coenzyme factor (for which we are indebted to Dr Straub of the Molteno Institute, Cambridge) the Q_{MB} rose to 99. Coenzyme factor is thus an essential component of the system and is normally present in juice *A* unless special measures are taken to destroy it.

Coenzyme I in the bacterial cell. Since coenzyme I is required for the functioning of the malic dehydrogenase of the organism, it is obvious from Table II that the cells as washed off agar contain some, but not an amount optimum for the activity of the malic dehydrogenase present. (Reduction time with cozymase = 23 min.; without cozymase = 68 min.) Lwoff & Lwoff [1937] showed that the *V* factor for *H. parainfluenzae* can be replaced by cozymase but that the organism grown in the presence of suboptimum amounts of coenzyme I dehydrogenates malate at a rate which can be increased if cozymase is added to the system. They studied the effect on the "index of codehydrogenase activity" of adding cozymase and its breakdown products to the growth medium and found that the organism is unable to synthesize coenzyme I from its various parts but requires the whole molecule supplied as such. To study whether a similar effect is obtained with *Bact. coli*, we adopted the "index of coenzyme saturation" ("i.c.s.") defined

$$\frac{\text{Activity without coenzyme I added}}{\text{Activity in presence of excess coenzyme I}} \times 100.$$

This can be regarded as showing the degree to which the organism is saturated with coenzyme in respect of the malic dehydrogenase.

In order to saturate the system in the case of malic dehydrogenase of *Bact. coli* grown on agar, it was necessary to add cozymase preparation equivalent to about 6 µg. coenzyme I per mg. dry weight bacteria to the washed suspension.

Before investigating the effect of growth conditions on the "i.c.s." it was necessary to determine whether this value varies in the washed suspension on standing and also whether it can be altered by incubating the washed suspension in the presence of cozymase.

Table VI. Variation of "i.c.s." on (a) incubation alone, and
(b) incubation with cozymase

Bacterial suspension 7.2 mg./ml. Tubes prepared as before, Table II.

Reduction time in presence of excess cozymase	— 24 min. 52 sec.
Reduction time in absence of cozymase	— 63 min. 50 sec.
Index of codehydrogenase saturation	— 27

Treatment	"i.c.s."
Original washed suspension	27
Incubated alone 24 hr. at 37°	27
Incubated 3 hr. at 37° with cozymase, see text	34
Stood 3 hr. at 0° with cozymase	29

Table VI shows that incubation of a washed suspension of the organism for 24 hr. produces no change in the value of the "i.c.s." At the same time 6 ml. of the bacterial suspension were incubated with 8 mg. cozymase preparation for 3 hr. It was then centrifuged, the cells washed once and the "i.c.s." determined. A small rise—27 to 34—was obtained but since cell division probably commenced during that time and, as will be shown later, growth in the presence of cozymase

results in an increase of "i.c.s.", this rise is probably not significant. Thus the "i.c.s." of the washed non-dividing suspension is constant and is not altered by incubation alone or in the presence of cozymase.

Action of Bact. coli on cozymase I. Euler & Gunther [1936], Euler & Heiwinkel [1937] and Euler *et al.* [1937] have shown that cozymase is inactivated by certain animal and vegetable tissues. That this is not the case with *Bact. coli* was shown as follows: 5 test tubes were set up as follows:

Tube	1	2	3	4	5
Cozymase 2 mg./ml.	1 ml.	1 ml.	1 ml.	—	—
Bacterial suspension 5 mg./ml.	1 ml.	—	—	1 ml.	—
Boiled bacterial suspension 5 mg./ml.	—	1 ml.	—	—	1 ml.
Water	—	—	1 ml.	1 ml.	1 ml.

The tubes were incubated aerobically for 4 hr. at 37°, then boiled for 10 min. and 1 ml. from each tested for coenzyme activity, using the malic dehydrogenase preparation from *Bact. coli*. The results in Table VII show that the cozymase in tube 1, incubated in the presence of the organism, has suffered no appreciable inactivation in 4 hr. as the amount of coenzyme used is insufficient to saturate the enzyme even before treatment.

Table VII. *Action of Bact. coli on cozymase*

Tubes prepared as before with regard to additions (a)–(d). 1 ml. of juice A used as a source of malic dehydrogenase. 1 ml. from each of tubes 1–5 (see text) used in turn as source of coenzyme.

Coenzyme addition from tube no.	Reduction time
None	> 3 hr.
1	8 min.
2	8 min.
3	8½ min.
4	> 3 hr.
5	> 3 hr.

Variation of "i.c.s." with age of culture. Cultures were grown in caseinogen tryptic broth in Roux bottles and prepared in washed suspension at various periods during their growth. In each case the activity of the malic dehydrogenase in the absence of added coenzyme (curve 1), the activity in the presence

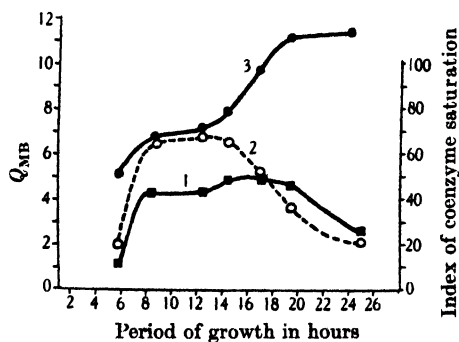


Fig. 3. Variation with age of culture of (1) enzyme activity (Q_{MB}) without added cozymase; (2) index of coenzyme saturation; (3) enzyme activity in presence of excess cozymase I.

of excess coenzyme (curve 3) and the "i.c.s." calculated from these results (curve 2) were determined and the results are shown in Fig. 3. Curve 3 is a measure of the amount of enzyme formed, while curve 1 is the amount of that

enzyme activated by the coenzyme present in the organism. From this it appears that young cultures have relatively little coenzyme I and that the enzyme content increases until growth ceases, whilst the increase in coenzyme remains stationary after 8 hr. so that the "i.c.s." falls after the 14th hr. Wooldridge *et al.* [1936] and Wooldridge & Glass [1937] have shown that the activity of certain dehydrogenases varies during the growth period with *Bact. coli* and that, in general, this variation consists of an increasing activity during the early part of growth and a falling off in activity towards the end of active cell division. This is seen to be the case in curve 1 but, in this case, the apparent fall in activity is due to an insufficient amount of coenzyme synthesized by the organism.

Since there is an increase in "i.c.s." from the 6th to the 8th hr. of growth, one may deduce that the organism can synthesize coenzyme I during that period and the question arises whether the later fall in the "i.c.s." is due to a loss of this power or to the exhaustion of some essential factor in the medium which the organism is unable to synthesize with sufficient rapidity.

Variations in "i.c.s." by alterations in and additions to the growth medium. In the following experiments the organisms were harvested at the end of 24 hr. when the "i.c.s." has normally fallen to a value of 20–30. The media used were as follows:

1. Stephenson's inorganic medium + 2 % lactate + 2 % aspartate.
2. Tryptic broth agar.
3. Tryptic broth (strictly anaerobic).
4. Broth in Roux bottles.
5. As (4) + *M*/3300 adenine.
6. " *M*/3300 adenosine.
7. " *M*/3000 ribose.
8. " *M*/3000 nicotinamide.
9. " *M*/3000 each of adenosine, ribose, nicotinamide.
10. " 0.0002 % coenzyme I.
11. " 0.0005 % coenzyme I.

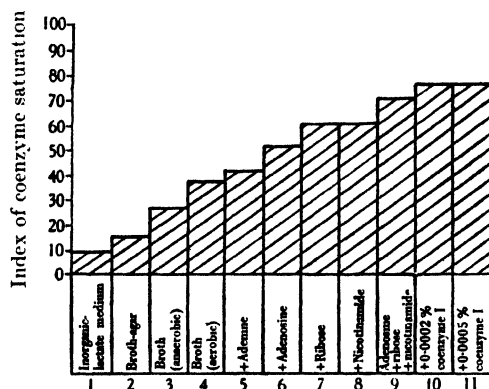


Fig. 4. Effect of additions to growth medium on the "i.c.s." of 24 hr. cultures.

In each case the "i.c.s." of the washed suspension was determined and the results are shown diagrammatically in Fig. 4. (1) shows that the power to synthesize coenzyme I from a simple medium is very restricted, about 4 times as much being synthesized in the caseinogen tryptic broth (4). The fall in "i.c.s."

shown in Fig. 3 is checked by the addition to the medium of coenzyme I (10) but the "i.c.s." never seems to reach 100, as increasing the quantity of coenzyme added to the medium (11) has no further effect. Addition of the various components of coenzyme I either singly or together does increase the value of the "i.c.s." to varying extents as shown in Fig. 4; ribose and nicotinamide being about equally effective while adenine has little effect.

These results suggest that the fall of "i.c.s." towards the end of growth in tryptic digest of casein is due to the exhaustion of some factor or factors used by the organism for the synthesis of coenzyme I which it is unable to synthesize with any rapidity for itself.

Oxidation of l-malate by Bact. coli

A washed suspension of *Bact. coli* when shaken aerobically with *l*-malate in the presence of buffer, absorbs O_2 . The O_2 uptake has been studied in Warburg manometers containing 1 ml. suspension of organism (dry wt. 1.5–2.0 mg./ml.), 1 ml. phosphate buffer pH 7.2 and 0.4 ml. *M*/60 Na malate in the main compartment and 0.2 ml. 10% NaOH in the centre cup. When corrected for the blank respiration of the organism, the malate is oxidized at a rate $Q_{O_2} = 220$. Complete oxidation of malate would require 6 atoms of O per molecule. In the above experiment the total O_2 uptake amounted to 307 μ l. which corresponds to an uptake of 4 atoms of O per molecule of malate; thus the oxidation is not carried to completion. With oxaloacetic acid as substrate, the organism carries out an oxidation corresponding to an uptake of 3 atoms O per molecule with a $Q_{O_2} = 220$.

The addition of cozymase to the reaction mixture has no effect on either the Q_{O_2} or the total O_2 consumption for the oxidation of malate. Since the addition of cozymase has a marked effect on the dehydrogenase activity of the organism but not on the Q_{O_2} it follows that the rate of O_2 consumption is not limited by the velocity of reaction of the dehydrogenase: this is confirmed by the fact that oxaloacetic acid gives the same Q_{O_2} as that for malate and it will be shown later that oxaloacetic acid is the product of the dehydrogenation of malic acid.

The oxidation of malate by the whole organism is completely inhibited by the presence of 10^{-4} *M* cyanide. Of other keto-fixatives tried, semicarbazide (*M*/100) is without effect; hydroxylamine (*M*/500) is toxic and hydrazine is itself partially oxidized.

Oxidation of l-malate by malic dehydrogenase extracted from Bact. coli

The enzyme was extracted from the washed suspension of organism as described above and the source used throughout the following experiments was the juice *A* obtained by centrifuging the crushed material for 30 min. at 3000 r.p.m. To obtain oxidation of malate by the juice in air, the following components are necessary:

malate—enzyme—coenzyme I—coenzyme factor—cyanide—methylene blue— O_2 .

It has been shown that the coenzyme factor is contained in the juice *A* with the dehydrogenase. Figs. 5–8 show the effect of varying the concentration of the various components on the rate of O_2 consumption, using 1 ml. of juice *A* as the source of enzyme in each case. The extracted enzyme has a low affinity for malate, the optimum concentration being *M*/14 (Fig. 5). The work previously described has shown that coenzyme I is required by the system and that juice *A* contains insufficient to give rise to appreciable activity. Fig. 6 shows that maximum activity is obtained with the enzyme preparation by the addition

of 150 $\mu\text{g.}$ coenzyme I/ml. preparation. The system will not react with O_2 in the absence of a carrying system which can be supplied by a high concentration of

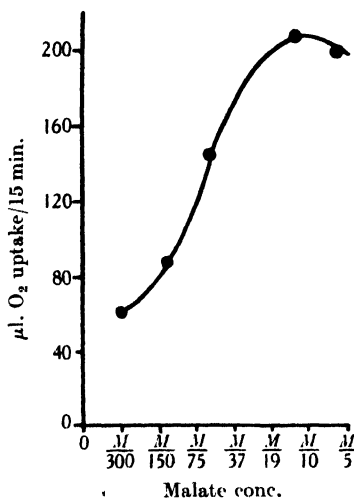


Fig. 5.

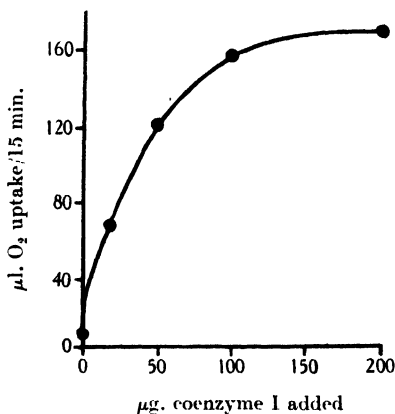


Fig. 6.

Fig. 5. Effect of substrate concentration. Manometers contain 1 ml. juice *A*; 0.5 ml. $M/5$ phosphate buffer; 0.5 ml. 0.5% MB; 0.5 ml. cozymase; 0.1 ml. 2 M NaCN.

Fig. 6. Effect of coenzyme concentration. Manometers contain 1 ml. juice *A*; 0.5 ml. $M/5$ phosphate buffer; 0.3 ml. $M/2$ malate; 0.5 ml. 0.5% MB; 0.1 ml. 2 M NaCN.

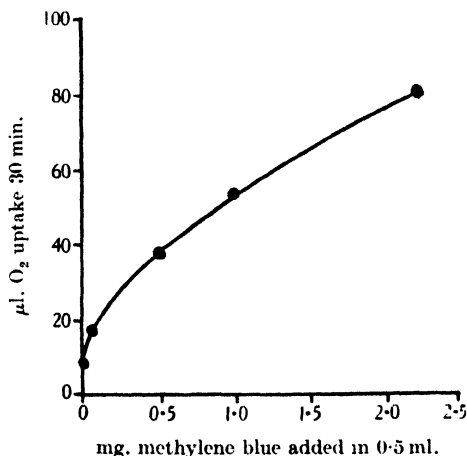


Fig. 7.

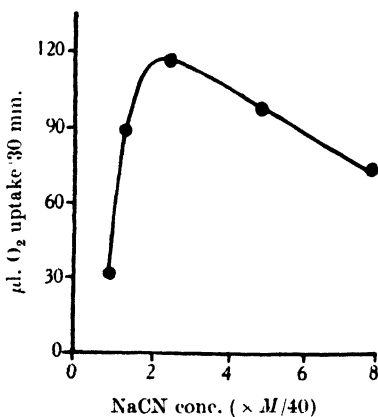


Fig. 8.

Fig. 7. Effect of methylene blue concentration. Manometers contain 1 ml. juice *A*; 0.5 ml. $M/5$ phosphate buffer; 0.5 ml. cozymase; 0.1 ml. 2 M NaCN; 0.3 ml. $M/2$ malate.

Fig. 8. Effect of cyanide concentration. Manometers contain 1 ml. juice *A*; 0.5 ml. cozymase; 0.2 ml. 0.1% MB; 0.3 ml. $M/2$ Na malate; 0.5 ml. $M/5$ phosphate buffer.

MB (Fig. 7). Further, as Green [1936] has shown, the malic dehydrogenase is inhibited by the oxaloacetic acid formed and an O_2 uptake cannot be obtained unless a keto-fixative is present. HCN is the most efficient fixative and has

an optimum concentration, under the experimental conditions, of *M*/16 (Fig. 8). Semicarbazide, hydroxylamine, hydrazine and bisulphite proved ineffective in this case, no significant O_2 consumption being obtained in their presence.

The malic dehydrogenase extracted from *Bact. coli* thus differs from that extracted from heart muscle by Green [1936] in its reactions with O_2 , in being inhibited by high concentrations of cyanide (Fig. 8) and in being unable to react in the presence of semicarbazide, hydroxylamine or hydrazine.

Table VIII shows the O_2 uptake obtained with the complete system and that no reaction is obtained if any one of the components is missing. The O_2 uptake under optimum conditions is not linear, the Q_{O_2} being halved in about 20 min. This effect cannot be altered by reducing the O_2 tension as has been shown by Gale [1939] for formic dehydrogenase. Owing to the low affinity of the extracted enzyme and the rapid deterioration of its activity, it is difficult to obtain the theoretical O_2 uptake. If the cyanide acts by fixing the oxaloacetic acid formed by the dehydrogenation, then an O_2 consumption should be obtained corresponding to an uptake of 1 atom O_2 per molecule malate. Fig. 9 gives an experiment in which this was realized.

Table VIII. *Oxidation system for extracted malic dehydrogenase*

Manometer	1	2	3	4	5
	Additions in ml.				
Juice <i>A</i>	1	1	1	1	1
<i>M</i> /5 phosphate pH 7.2	0.5	0.5	0.5	0.5	0.5
<i>M</i> /2 Na malate	—	0.3	0.3	0.3	0.3
0.5% methylene blue	0.5		0.5	0.5	0.5
2 <i>M</i> NaCN (neutral)	0.1	0.1	—	0.1	0.1
Cozymase 2 mg./ml.	0.5	0.5	0.5		0.5
Water	0.3	0.5	0.1	0.5	—
μ l. O_2 uptake/15 min.	0	14	0	0	196

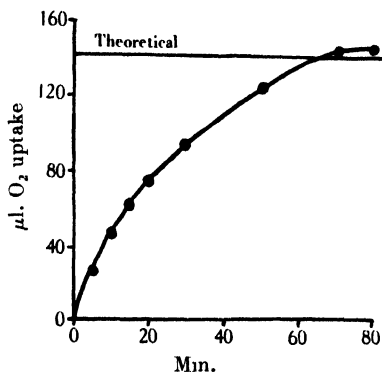


Fig. 9. Oxidation of *l*-malate by extracted enzyme system showing uptake of 1 atom of O /mol. malate oxidized. Manometer contains 1 ml. juice *A*; 0.5 ml. *M*/5 phosphate buffer; 0.5 ml. 0.5% MB; 0.5 ml. cozymase; 0.1 ml. 2 *M* NaCN and 0.5 ml. *M*/40 Na malate.

Product of the oxidation. Since cyanide is the only fixative which will allow of an appreciable rate of reaction, the keto-acid produced by the dehydrogenase has to be obtained first as the cyanohydrin. An experiment was set up as follows: 30 ml. of juice *A* were mixed with 20 ml. 0.5% MB, 3 ml. 2 *M* NaCN and 25 ml. *M*/5 phosphate buffer at pH 7.2 in which were dissolved 1.5 g. malic acid (neutralized) and 100 mg. cozymase preparation. The whole was divided into three

lots and incubated for 2 hr. at 37°, O₂ being bubbled throughout. At the end of that time the material was collected and cooled in ice. It was then deproteinized by treatment with colloidal Fe(OH)₃ and filtration through kieselguhr. Final traces of methylene blue were removed by shaking with a little kieselguhr followed by filtration.

The cyanohydrin will not react with 2:4-dinitrophenylhydrazine and warming with acid or alkali results in its decomposition. The procedure finally adopted [cf. Green & Williamson, 1937] was to add 10% NaOH until the final concentration was *N*/5 NaOH, and after leaving in ice for 5 min. quickly pour into an acid solution of 2:4-dinitrophenylhydrazine (0.5%) containing sufficient HCl to make the final acidity 2*N*. The mixture was then left to stand at room temperature for 24 hr. when the 2:4-dinitrophenylhydrazone slowly crystallized and was filtered off (crude yield 120 mg.). The substance was recrystallized [Clift & Cook, 1932] by dissolving the dry crystals in the minimum ethyl acetate, adding ligroin until turbid and leaving to crystallize. After two recrystallizations by this method, it was washed with ligroin and dried *in vacuo*: M.P. 212° decomp. Found (Weiler): C, 37.93%; H, 3.04%; N, 17.8%. Calc. for oxaloacetic 2:4-dinitrophenylhydrazone: C, 38.34%; H, 2.58%; N, 17.9%.

Reversibility of the dehydrogenase. It has been shown that the extracted enzyme reduces coenzyme I when incubated in the presence of malate. It remains to show the oxidation of reduced coenzyme I in the presence of the enzyme and oxaloacetic acid.

Reduced coenzyme I was prepared as follows: 30 mg. cozymase preparation (15% coenzyme I) were dissolved in 10 ml. *M*/2 NaHCO₃, 20 mg. Na₂S₂O₄ crystals added and the whole incubated anaerobically for 30 min. The solution was then aerated vigorously for 15 min. to oxidize the excess hydrosulphite. 1 ml., diluted 1/5, was taken for estimation by means of the Hilger Spekker spectrophotometer. $\log I_0/I = 1.0$ at 345 m μ .

4 ml. of the reduced coenzyme solution were then incubated with 2 ml. juice *A* and 2 ml. *M*/40 Na oxaloacetate at 37° *in vacuo* for 20 min. The reaction mixture was then brought to the boil, the protein filtered off, the filtrate diluted 2/5 and its spectrum examined. $\log I_0/I = 0.5$ —the absorption in this case being due to a non-specific absorption in the reaction mixture. A control performed with boiled enzyme and reduced coenzyme gave a well defined absorption band at 345 m μ , $\log I_0/I = 0.9$.

Thus incubation of reduced coenzyme with the enzyme in the presence of oxaloacetate results in oxidation of the reduced coenzyme.

SUMMARY

1. Washed suspensions of *Bact. coli* contain malic dehydrogenase.
2. When suspensions of *Bact. coli* are crushed in the Booth-Green mill, the malic dehydrogenase is extracted from the cell and resides mainly in the liquid fraction of the crushed material.
3. The malic dehydrogenase of the *coli*-juice requires the addition of coenzyme I and coenzyme factor (diaphorase) for its action with methylene blue.
4. When suspensions of *Bact. coli* are boiled, coenzyme I is liberated from the cell and passes into the supernatant fluid obtained by centrifuging.
5. *Bact. coli* does not inactivate cozymase at a significant rate when incubated with it.
6. Organisms grown on agar or in tryptic broth do not contain the optimum amount of coenzyme to saturate their malic dehydrogenase.

7. The "index of coenzyme saturation" varies with the age of the culture, young cultures having a low value (20 %) which rises by the 10th hr. of growth to 65 %, remains steady until the 14th hr. and then falls until growth ceases—having a value of 20 % by the 24th hr.

8. Addition of coenzyme I and, to a smaller extent, ribose and/or nicotinamide to the growth medium prevents this fall of "i.c.s." with age of culture.

9. *Bact. coli* oxidizes *l*-malate aerobically, taking up 4 atoms of O per molecule malate oxidized. The oxidation is inhibited by 10^{-4} M cyanide.

10. The malic dehydrogenase extracted from the cells will oxidize *l*-malate aerobically in the presence of the following system: malate—dehydrogenase—coenzyme I—coenzyme factor—cyanide—methylene blue—O₂. The effect of variation of concentration of these factors is studied.

11. Under such conditions 1 atom of O is taken up per molecule of malate oxidized and the product of the oxidation is oxaloacetic acid, which has been isolated as the 2:4-dinitrophenylhydrazone.

12. The dehydrogenase is reversible, reduced coenzyme I being oxidized by the enzyme in the presence of oxaloacetic acid.

The authors wish to express their thanks to Dr D. E. Green for his advice and to Prof. Sir F. G. Hopkins for his interest in this work.

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CLIV. THE SPECIFICITY OF THE ACTION OF LACTIC ACID BACTERIA ON THE PHOSPHOGLYCERIC ACIDS

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THE specificity of the form of the 2- and 3-phosphoglyceric acids decomposed by lactic acid bacteria has not yet been demonstrated, although the production of phosphoglycerate from glucose was recently proved by Stone & Werkman [1937] with *Lactobacillus pentoaceticus* and *Lactobacillus plantarum*, and Neuberg & Kobel [1933] ascertained the decomposition of phosphoglycerate into pyruvic acid by *Bacillus delbrückii*.

In the present work experiments were carried out with various strains of lactic acid bacteria, in order to examine whether any selective decomposition of phosphoglycerates occurred and whether the racemization of phosphoglycerates by racemase took place.

Methods

Ba *dl*-2- and Ba *dl*-3-phosphoglycerates were prepared by the methods described by Kiessling [1935] and by Neuberg *et al.* [1928] respectively, and the purifications of these Ba salts were in each case repeated several times until their Ba and P contents had attained nearly the theoretical values.

The Ba salts were dissolved in acetic acid, the Ba removed by 10 % Na₂SO₄ and the pH brought to 6.3 by addition of 33 % NaOH.

Lactobacillus plantarum (*dl*-acid-former), *Leuconostoc mesenteroides* var. *sake* (*l*-acid-former) and *Lactobacillus sake* (*d*-acid-former) isolated in our laboratory were used in the experiments.

The bacteria were cultivated at 30° for 4 days in flasks containing a medium consisting of 600 ml. koji extract containing 24–30 g. sugar and 30 g. CaCO₃. The bacterial cells were collected and washed with water on the centrifuge, after being separated from CaCO₃ by decantation. The fresh cells (nearly 1.8 g. dry matter) were suspended in about 50 ml. water.

The bacterial suspension was added to about 80 ml. Na phosphoglycerate solution prepared from 2–3 g. Ba salt, toluene was added and the mixture diluted with water up to 150–200 ml.

After being kept at about 30° for 0.5–3 hr., 50 ml. 40 % trichloroacetic acid were added and the mixture centrifuged. The supernatant liquid was concentrated under reduced pressure and neutralized by NaOH after the addition of 5 g. Ba acetate; the phosphoglycerate was then recovered from the solution as crude Ba salt by the addition of two volumes of alcohol.

The crude Ba salt was dissolved in dil. HCl, acidified with 2*N* H₂SO₄ and filtered. The inorganic phosphate in the solution was precipitated by the addition of NH₄OH and then magnesia mixture. The dephosphorylated solution was again acidified to Congo red by HNO₃ and 25 % Ba acetate was added. The filtrate was made weakly acid to litmus with NaOH and the Ba salt precipitated by alcohol.

The Ba salt thus obtained was purified 2-3 times until crystalline precipitates of nearly 90-98 % purity were obtained, by dissolving in dil. HCl which gave a solution faintly acid to Congo red and then adding alcohol drop by drop. The yield recovered was calculated by weighing these crystalline salts, $C_3H_5O_7PBa \cdot 3H_2O$, and the rotatory power (calculated as $[\alpha]_D^{20}$) was determined with its solution in *N* HCl in the 2 dm. tube.

In the case of yeast maceration extract prepared from dried beer yeast, 150 ml. of the extract were first neutralized with *M*/2 Na_2CO_3 and then added to 50 ml. 6 % Na phosphoglycerate, and the experiments were carried out in the presence of toluene.

dl-Phosphoglycerates

By the action of yeast maceration extract upon Na *dl*-2- or -3-phosphoglycerate for 2 hr. at 28° and by the action of fresh cells of various strains of lactic acid bacteria on the 2-phosphoglycerate for 3 hr. at 28° and on the 3-phosphoglycerate for 2.5 hr. at 27°, part of the phosphoglycerate was always decomposed as will be seen in the yields of recovery of the acids given in Table I, and the production of pyruvic acid was observed in all the reacting solutions by the method described by Case [1932].

Table I. *Selective decomposition of dl-2- and -3-phosphoglycerates*

Organism	2-Phosphoglyceric acid		3-Phosphoglyceric acid	
	Yield of recovery %	$[\alpha]_D^{20}$	Yield of recovery %	$[\alpha]_D^{20}$
Yeast maceration extract	45-50	- 23.33	40-45	+ 18.66
<i>dl</i> -Acid-former	30	- 0.73	35	- 0.80
<i>l</i> -Acid-former	40	- 23.98	30	- 14.34
<i>d</i> -Acid-former	40	+ 26.45	30	+ 18.66

As will be seen from Table I, the optical forms of the recovered phosphoglyceric acids were in agreement with those of the lactic acids produced by the organisms which were employed: (-)-2-phosphoglyceric acid (theor. $[\alpha]_D^{20} - 24.3^\circ$) was recovered with yeast and *l*-acid-former, (+)-2-acid with *d*-acid-former; (+)-3-acid (theor. $[\alpha]_D^{20} + 14.5^\circ$) with yeast and *d*-acid-former, (-)-3-acid with *l*-acid-former, and with *dl*-acid-former the *dl*-forms of 2- and 3-phosphoglyceric acids were obtained.

Thus it was ascertained that yeast and *d*- and *l*-acid-formers decomposed phosphoglycerates selectively, while no optical specificity was found with the *dl*-acid-former.

Optically active 2-phosphoglycerates

The products of decomposition of *dl*-2-phosphoglycerate by a *d*- or *l*-acid-former were carefully purified. With these optically active Ba salts experiments were carried out in the same manner as was mentioned above.

The selective decomposition of the 2-phosphoglycerates by the bacterial cells is clearly shown in Table II: (-)-2-phosphoglyceric acid was decomposed by the *d*-acid-former but never by the *l*-acid-former with which only the optical antipode ((+)-isomeride) was easily attacked.

The results of the experiments shown in Table II not only showed that both forms of 2-phosphoglycerate were decomposed by the *dl*-acid-former as would be expected from the results shown in Table I, but also suggested that the bacterial cells caused racemization of the 2-phosphoglycerate, since the *dl*-form of

2-phosphoglyceric acid was always obtained from optically active acids by the actions of the *dl*-acid-former.

Table II. *Decomposition of (+)- and (-)-2-phosphoglyceric acids*

Lactic acid bacteria	Period of reaction hr.	Temp. of reaction	Yield of recovery %	$[\alpha]_D^{20}$
(-)-2-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	26.5	19.00	- 1.67
<i>l</i> -Acid-former	2.5	26	98.56	- 22.50
<i>d</i> -Acid-former	3.0	26	20.00	- 22.50
(+)-2-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	27	22.13	+ 1.67
<i>l</i> -Acid-former	2.5	26	21.21	+ 26.67
<i>d</i> -Acid-former	-	-	-	-

Optically active 3-phosphoglycerates

With the optically active 3-phosphoglycerates prepared from the *dl*-compound by the action of *d*- and *l*-acid-formers, the experiments shown in Table III were carried out.

Table III. *Decomposition of (-)- and (+)-3-phosphoglyceric acids*

Lactic acid bacteria	Period of reaction hr.	Temp. of reaction	Yield of recovery %	$[\alpha]_D^{20}$
(-)-3-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	30	52.43	- 0.83
<i>l</i> -Acid-former	2.5	26	93.32	- 13.33
<i>d</i> -Acid-former	0.5	25.5	79.86	- 13.33
(+)-3-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	30	46.88	+ 0.83
<i>l</i> -Acid-former	2.5	25.5	15.04	+ 14.17
<i>d</i> -Acid-former	2.5	26	95.96	+ 15.83

It is clearly seen from Table III that the *l*-acid-former decomposed only (+)-3-phosphoglyceric acid and the *d*-acid-former only (-)-3-phosphoglyceric acid, since (+)- and (-)-3-phosphoglyceric acids were never attacked by *d*- and *l*-acid-formers respectively.

With the *dl*-acid-former, both forms of 3-phosphoglyceric acid were decomposed and *dl*-3-phosphoglycerate was always found to be produced from any form of 3-phosphoglyceric acid.

Thus it was clearly demonstrated that the *l*-acid-former decomposed only (+)-2- and (+)-3-phosphoglyceric acids, whereas the (-)-2- and (-)-3-isomerides were selectively attacked by the *d*-acid-former.

These specificities of the lactic acid bacteria are in disagreement with that of yeast maceration extract which was found to effect the same selective decomposition of 2- and 3-phosphoglyceric acids as that observed for muscle extract by Meyerhof & Kiessling [1935] and by Kiessling & Schuster [1938].

When the *d*-acid-former was compared with muscle extract by which *d*-lactic acid would be produced, the specificity of 2-phosphoglycerate was found to be quite different, although the same (-)-form of 3-phosphoglyceric acid was selectively decomposed by the muscle and the *d*-lactic acid-former.

It is therefore suggested that Meyerhof's scheme of lactic acid formation, based upon experiments with muscle extract, would have to be modified as regards the behaviour of the phosphoglycerates before it could be applied to the fermentations produced by lactic acid bacteria.

Action of racemase upon phosphoglyceric acids

From the experimental results mentioned above, it was concluded that all the forms of 2- and 3-phosphoglycerates were decomposed by the *dl*-acid-former and it was suggested that *dl*-phosphoglyceric acids were always obtained from any one of the optically active components of 2- and 3-phosphoglyceric acids by the action of the bacterial cells.

When 0.4 g. Ba salt of the *dl*-product obtained from (+)-3-phosphoglyceric acid by the *dl*-acid-former (see Table III) was decomposed by *d*-acid-former, 0.0318 g. Ba salt of (+)-3-phosphoglyceric acid was produced as was expected from the results shown in Table I, since the specific rotation of the active salt was observed to be $+707.5^\circ$ (theor. $[\alpha]_D^{20} + 745^\circ$) by the molybdate method described by Meyerhof & Schulz [1938].

Therefore it is suggested that the *dl*-acid-former can catalyse racemization of 2- and 3-phosphoglyceric acids.

In order to ascertain whether the racemization of these phosphoglyceric acids would be effected by racemase in the bacterial cells, as had been already observed by us [1936; 1937; 1938, 1, 2] with the lactic acids, experiments were carried out at 30° for 2.5 hr. with the resting cells and with the maceration extract of dried cells of the *dl*-acid-former which were prepared as follows: 3.5 g. dried bacteria, dehydrated under reduced pressure over H_2SO_4 , were added to 15 ml. water, kept at 30° for 3 hr. in the presence of toluene and centrifuged.

Table IV shows that the optical properties of the recovered phosphoglyceric acids were quite different in the two cases, since (–)-2- and (–)-3-phosphoglyceric acids were obtained with the maceration extract as was observed with the *l*-acid-former, whereas *dl*-phosphoglyceric acids were always obtained by the resting cells as was mentioned above.

Table IV. *Effect of racemase*

	<i>dl</i> -2-Phosphoglyceric acid		<i>dl</i> -3-Phosphoglyceric acid	
<i>dl</i> -Acid-former	Yield of recovery %	$[\alpha]_D^{20}$	Yield of recovery %	$[\alpha]_D^{20}$
Resting cells	30	– 0.73	35	– 0.8
Maceration extract	28.2	– 25.00	21.3	– 16.66

It is worthy of note that the same (–)-2-phosphoglyceric acid ($[\alpha]_D^{20} - 23.33^\circ$) was recovered when the maceration extract of the *dl*-acid-former was allowed to act at 30° for 2 hr. on (–)-2-phosphoglyceric acid, whilst the racemic product was obtained with the resting cells (see Table II).

Thus it was ascertained that the action of racemase was not limited to lactic acids, since the racemization of 2- and 3-phosphoglycerates was effected by the enzyme.

SUMMARY

1. Selective decomposition of phosphoglycerates was observed with yeast maceration extract, a *d*-acid-former (*Lactobacillus sake*) and a *l*-acid-former (*Leuconostoc*).

2. With yeast maceration extract (+)-2- and (-)-3-phosphoglyceric acids were decomposed, a *l*-acid-former decomposed (+)-2- and (+)-3-phosphoglyceric acids and with the *d*-acid-former (-)-2- and (-)-3-phosphoglyceric acids were decomposed.

3. No optical specificity was found with resting cells of a *dl*-acid-former (*Lactobacillus plantarum*) by which all the forms of 2- and 3-phosphoglyceric acids were decomposed, while the same specificity as that of the *l*-acid-former was observed with maceration extract of the *dl*-acid-former.

4. It was found that racemization of 2- and 3-phosphoglyceric acids was produced by racemase.

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CLV. ENZYMIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUES

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Soon after the discovery by Lohmann & Schuster [1937, 1] that cocarboxylase is the pyrophosphate of vitamin B₁, there were attempts by various workers to show that the vitamin can be enzymically phosphorylated to cocarboxylase by yeast and tissue preparations. Positive results were claimed with rat liver extracts by Euler & Vestin [1937], with acetone-dried preparations from pig's duodenal mucosa by Tauber [1937], and with avitaminous brain brei by Peters [1937, 1]; whereas Stern & Hofer [1937] failed to obtain synthesis using glycerol extracts from various rat tissues (liver, brain, intestine). Later Lohmann & Schuster [1937, 2] also reported some synthesis with minced pigeon's brain. In all these experiments tissue samples were incubated with vitamin B₁ in phosphate buffer, boiled and approximately assayed for cocarboxylase by measurement of the CO₂ evolution with alkaline-washed yeast and pyruvate.

The finding that free vitamin B₁, whilst unable to replace cocarboxylase, strongly stimulates its action in yeast [Ochoa, 1938], invalidated the conclusions drawn from those experiments where comparison had been made of the activities of samples incubated without and with vitamin B₁ respectively [Euler & Vestin, 1937; Peters, 1937, 1] as the higher activity of the latter could be due to the effect of the vitamin on the yeast system itself. Later experiments, to be reported in this paper, made Tauber's claim [1937] untenable that synthesis occurs with acetone preparations of intestinal mucosa. More recently Goodhart & Sinclair [1939] have shown that blood from avitaminous pigeons can synthesize cocarboxylase from vitamin B₁.

Ochoa & Peters [1938, 1] developed a method for the determination of cocarboxylase with yeast in the presence of an excess of vitamin B₁; this not only eliminated the effect of any vitamin present in the samples but also increased the sensitivity of yeast to cocarboxylase about ten times. Using this method they showed that 30 min. after injecting vitamin B₁ into avitaminous pigeons and rats there is a marked accumulation of cocarboxylase in the liver whereas little or no increase occurs in muscle, heart or brain. The *in vivo* synthesis in liver was confirmed (with the thiochrome method) by Westenbrink & Goudsmit [1938] who also found synthesis in the kidney.¹ Curiously enough the cocarboxylase in liver, after the injection, reaches values which are not much above the normal; hence injection of vitamin into normal pigeons causes only slight increases of cocarboxylase [Ochoa & Peters, unpublished results]. This is why avitaminous birds have been mainly used in the experiments described in this paper, although good syntheses were occasionally obtained with normal tissues.

The *in vitro* experiments [preliminary report: Ochoa & Peters, 1938, 2] have confirmed the above results in their main lines and added further information as

¹ This has been confirmed in experiments on rats in this laboratory [Ochoa & Rossiter, unpublished].

to the mechanism of the enzymic synthesis of cocarboxylase. Some of the results with brain have already been reported in a previous paper [Banga *et al.* 1939]. The synthetic power of brain is very small indeed, yet it accounts fully for the cataturulin effect of vitamin B₁.

EXPERIMENTAL

The majority of the experiments were carried out with slices, brei or "dispersions" of tissues from avitaminous pigeons. In some cases normal birds or birds which had been on a rice diet ("rice-fed") for various periods, but did not yet show symptoms, were also used. The tissue was suspended in Ringer phosphate, generally 2 ml. of medium to 0.5 g. tissue. "Dispersions" were prepared by thoroughly grinding the minced tissue in an ice-cold mortar with 4 vol. of ice-cold Ringer phosphate; the mixture was pressed through muslin and the resulting fine suspension used. Sometimes the mixture was centrifuged, the resulting solution (extract) and the solid residue (suspended in Ringer) being used separately or the residue was resuspended in the extract.

The samples plus additions were shaken, mostly at 38°, either in 25 ml. conical flasks, or in Warburg bottles when O₂ uptake was being measured. After incubation the samples were heated to 100° for 2-3 min., cooled, centrifuged and the cocarboxylase determined in the centrifugate as described by Ochoa & Peters [1938, 1]. Control samples were incubated without vitamin B₁, which was only added at the end (in the maximum amount used in the experiment) just before boiling.

No attempt was made to provide for buffering when working at reactions more alkaline than pH 8.0. The Ringer phosphate was merely brought to the desired pH with NaOH, as it seemed preferable to avoid the use of buffers other than phosphate. Pyrophosphate was found to inhibit the cocarboxylase synthesis.¹

Results are expressed in μ g. cocarboxylase per g. fresh tissue or its equivalent when extracts, acetone powder preparations etc. are used.

Synthesis of cocarboxylase under various conditions

Table I shows the results of experiments with pigeon tissue preparations. The following points should be noted: (1) Of the various tissues tried (liver, brain, breast muscle, intestine) liver is the only one which shows a good capacity to phosphorylate added vitamin B₁ to cocarboxylase. (2) Liver slices, brei or "dispersion" show a similar degree of activity; liver extracts are much less active (exps. 12 and 13), whereas suspensions of acetone-dried liver, prepared after Tauber [1938], are inactive (exps. 13 and 14). (3) The synthesis of cocarboxylase is much reduced in the absence of O₂ (exps. 2 and 8). (4) The cocarboxylase synthesis reaches a maximum (which is generally not much higher than the amounts present in the normal tissue) beyond which it does not go, no matter how much vitamin B₁ is added. Thus in exp. 2 the same results are obtained with 20 as with 40 μ g. vitamin per g. tissue and 200 μ g. vitamin B₁, in exp. 16 produced no higher synthesis than 50 μ g. Hence the percentage of vitamin converted into cocarboxylase is higher with small amounts of the former. In exp. 3 the synthesis is only slightly lower with 10 μ g. than with 20 μ g. vitamin per g. tissue but the conversion is 28% in the former and only 18% in the latter case. (5) Addition of various substrates with the vitamin did not increase the extent of the cocarboxylase synthesis.

¹ This may be due to its depressing action on respiration [cf. Greig & Munro, 1939].

Table I. *Synthesis of cocarboxylase from vitamin B₁ in various preparations of pigeon's tissues*

Exp. no.	Condition of animals	Enzyme	pH	Time of incubation and temp.		Gas	Additions (per g. tissue)	Cocarboxylase $\mu\text{g. per g. tissue}$	
								Found	Synthesis
1	Avit.	Liver slices	7.3	1 hr.	38°	O ₂	Control 50 $\mu\text{g.}$ vitamin B ₁	0.40 5.10	— 4.70
2	Avit.	Liver slices	7.3	„	„	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 20 $\mu\text{g.}$ vitamin B ₁	0.50 4.90 5.15	— 4.40 4.65
						N ₂	40 $\mu\text{g.}$ vitamin B ₁	1.40	0.90
3	Avit.	Liver slices	7.3	„	„	O ₂	Control 20 $\mu\text{g.}$ vitamin B ₁ 10 $\mu\text{g.}$ vitamin B ₁	0.90 6.40 5.20	— 5.50 4.30
4	Normal	Liver slices	7.3	„	„	O ₂	Control 60 $\mu\text{g.}$ vitamin B ₁	2.60 6.10	— 3.50
5	Avit.	Brain slices	7.3	„	„	O ₂	Control 95 $\mu\text{g.}$ vitamin B ₁	0.25 0.55	— 0.30
6	Avit.	Muscle brei	7.3	„	„	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.85 1.47	— 0.62
7	Avit.	Intestine*	7.3	„	„	O ₂	Control 15 $\mu\text{g.}$ vitamin B ₁	0.12 0.28	— 0.16
8	Avit.	Liver slices	7.3	$\frac{1}{2}$ hr.	38°	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose	0.65 3.95 3.65	— 3.30 3.00
						N ₂	40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose	1.76 1.89	1.11 1.24
9	Avit.	Liver brei	7.3	„	„	O ₂	Control 50 $\mu\text{g.}$ vitamin B ₁ 50 $\mu\text{g.}$ vitamin B ₁ 45 $\mu\text{g.}$ ATP† 50 $\mu\text{g.}$ vitamin B ₁ 600 $\mu\text{g.}$ Pgl.‡ 50 $\mu\text{g.}$ vitamin B ₁ 45 $\mu\text{g.}$ ATP 600 $\mu\text{g.}$ Pgl.‡	1.46 4.22 4.22 3.48 3.22	— 2.76 2.76 2.02 1.76
10	Avit.	Liver slices	8.5	1 hr.	38°	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.52 5.50	— 4.98
		Liver brei	8.5	„	„	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.68 14.60	— 13.92
11	Avit.	Liver brei	8.5	„	„	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.50 7.80	— 7.30
12	Avit.	Liver extract	8.5	„	„	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose 40 $\mu\text{g.}$ vitamin B ₁ 90 $\mu\text{g.}$ ATP† 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose 90 $\mu\text{g.}$ ATP†	0.72 2.25 2.25 1.28 1.76	— 1.53 1.53 0.56 1.04

Table I (cont.)

Exp. no.	Condition of animals	Enzyme	pH	Time of incubation and temp.		Gas	Additions (per g. tissue)	Coccarboxylase $\mu\text{g. per g. tissue}$	
								Found	Synthesis
13	Avit.	Liver brei	8.5	1 hr.	38°	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	8.50	7.90
		Liver extract	8.5	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	2.10	1.50
		Liver acetone powder	8.5	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	0.70	0.10
14	Avit.	Liver acetone powder	8.5	O ₂	Control	0.40	—
							30 $\mu\text{g.}$ vitamin B ₁	0.40	0.00
15	Normal	Liver dispersion	8.5	O ₂	Control	3.40	—
							100 $\mu\text{g.}$ vitamin B ₁	13.40	10.00
16	Rice-fed	Liver brei	8.5	O ₂	Control	1.14	—
							50 $\mu\text{g.}$ vitamin B ₁	9.65	8.51
							100 $\mu\text{g.}$ vitamin B ₁	9.90	8.76
							200 $\mu\text{g.}$ vitamin B ₁	8.80	7.66
							200 $\mu\text{g.}$ vitamin B ₁	7.15	6.01
							20 mg. glucose		
							200 $\mu\text{g.}$ vitamin B ₁	6.36	5.22
							10 mg. NaHDP [†]		
							200 $\mu\text{g.}$ vitamin B ₁	6.88	5.74
							25 mg. Na succinate		

* Minced duodenum.

† Phosphoglyceric acid P.

‡ Easily hydrolysable P, adenosine triphosphate.

§ Na hexosediphosphate.

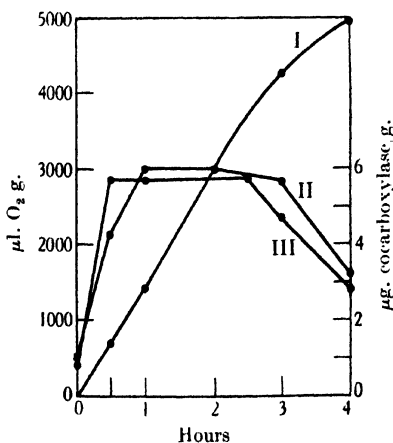


Fig. 1.

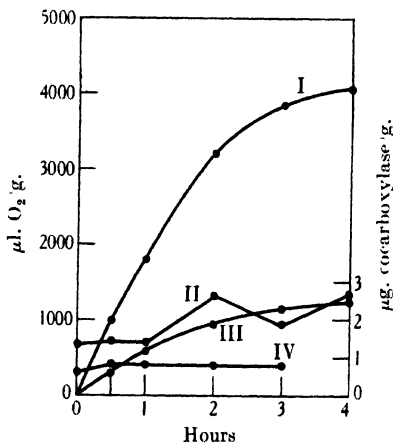


Fig. 2.

Fig. 1. O₂ uptake and synthesis of cocarboxylase from vitamin B₁ in liver from "rice-fed" pigeons. 250 mg. tissue in 1 ml. Ringer phosphate. 10 $\mu\text{g.}$ vitamin B₁ added at time 0. Curve I: O₂ uptake at 28° (liver brei); pH 8.5. Curve II: cocarboxylase at 28° (liver brei); pH 8.5. Curve III: cocarboxylase at 38° (liver slices); pH 7.3. Gas O₂.

Fig. 2. O₂ uptake and synthesis of cocarboxylase from vitamin B₁ in breast muscle and brain brei from "rice-fed" pigeons at 28°. 250 mg. tissue in 1 ml. Ringer phosphate pH 8.5. 10 $\mu\text{g.}$ vitamin B₁ added at time 0. Curve I: O₂ uptake (muscle). Curve II: cocarboxylase (muscle). Curve III: O₂ uptake (brain). Curve IV: cocarboxylase (brain). Gas O₂.

The formation of cocarboxylase in liver preparations reaches a maximum in 30–60 min. After remaining constant for a short time, the cocarboxylase later decreases. This is shown, for liver brei, in Fig. 1, both at 28 and 38°; the O₂ uptake was measured in the experiment at 28°. In Fig. 2 similar experiments with

brain and muscle are reproduced; here the cocarboxylase synthesis is much less and there is no drop from the maximum reached.

Experiments with liver slices at various *pH* are given in Table II. The synthetic reaction has a markedly alkaline *pH* optimum of about 8.5.

Table II. *Synthesis of cocarboxylase from vitamin B₁ in slices of liver from avitaminous pigeons at various pH*

40 μ g. vitamin B₁ per g. tissue added. Incubation in O₂, 40 min. at 38°.

Original <i>pH</i> of medium	Cocarboxylase synthesized (μ g. per g. tissue)		
	Exp. 1	Exp. 2	Exp. 3
6.2	3.32	—	3.47
6.8	4.09	—	—
7.3	5.08	—	—
8.0	5.42	8.31	5.07
8.5	—	12.51	5.51
9.0	—	10.51	—
9.5	—	11.41	5.07
10.0	—	5.21	4.24

Preparations from intestinal mucosa

Tauber [1937; 1938] claims to have obtained synthesis of cocarboxylase with acetone-dried preparations of duodenal mucosa from the pig. Exp. 7 of Table I shows that with pigeon's intestine hardly any synthesis is obtained. It has already been noted that acetone preparations of liver made by Tauber's method are inactive (Table I, exps. 13 and 14). In view of this, some experiments were done with pig's duodenal mucosa using the fresh minced mucosa, extract in Ringer phosphate and a suspension of the acetone-treated material after Tauber. In all cases the intestine from freshly slaughtered pigs was immediately brought into the laboratory on ice and the preparation of the mucosa was started at once. On incubation of the various preparations with vitamin B₁ for 1 hr. at 38° in O₂, at the optimum *pH* for liver (8.5), no synthesis of cocarboxylase was found (Table III). In his experiments Tauber [1937; 1938] used very long incubation periods of 20 hr. and more and found the optimum reaction to be

Table III. *Absence of cocarboxylase synthesis with preparations from pig's duodenal mucosa*

Incubation in O₂ at 38°, 1 hr. Medium: Ringer phosphate *pH* 8.5. 200 mg. acetone powder (= 2 g. fresh mucosa) in 4 ml. Ringer.

Preparation	Additions per g. tissue	Cocarboxylase (μ g. per g. tissue)	
		Found	Change
Brei	Control	0.52	—
	40 μ g. vitamin B ₁	0.50	- 0.02
Extract	Control	0.31	—
	40 μ g. vitamin B ₁	0.31	0.00
Acetone powder	Control	0.26	—
	40 μ g. vitamin B ₁	0.28	+ 0.02

approximately *pH* 6.8. He worked, as far as possible, under sterile conditions but it is impossible to sterilize the enzyme suspension satisfactorily. When Tauber's experiment was repeated a large synthesis of cocarboxylase was found to have taken place after 20 hr. incubation, but the samples taken at various intervals, up to 8 hr., showed no synthesis whatever; on the contrary, there was a slight and gradually increasing breakdown of the cocarboxylase originally

present (Table IV). This result indicates that the synthesis after 20 hr. must have been due to growing bacteria. If the mucosa preparation had contained an active enzyme, the synthesis of cocarboxylase would be expected to have started earlier and increase gradually.

Table IV. *Cocarboxylase in acetone preparations of pig's duodenal mucosa incubated for various periods with vitamin B₁*

Each sample contained 200 mg. acetone powder in 4 ml. 0.1 M phosphate buffer pH 6.8 with 0.4 mg. vitamin B₁ HCl. Incubation in air at 33°.

Incubation time hr.	Cocarboxylase (μg. per sample)	
	Found	Change
0	1.4	—
1½	0.7	- 0.7
3	0.6	- 0.8
5	0.5	- 0.9
8	0.4	- 1.0
20	26.0	+ 24.6

It is now well known that bacteria can synthesize cocarboxylase from vitamin B₁. This has been shown by the recent experiments of Silverman & Werkman [1939] and Barron & Lyman [1939]. Earlier experiments by Hills [1938] and Silverman & Werkman [1938] already indicated this possibility.

Relation of cocarboxylase synthesis to respiration

The experiments of Table I show that the synthesis of cocarboxylase is very small in liver extracts, and also in more active liver preparations if O₂ is absent. The low activity of the extracts is due to the fact that their O₂ uptake is very small, but the synthesizing enzyme is soluble and is present in them. The experiment of Table V shows this very clearly. The samples contained liver extract, the remaining solid residue suspended in Ringer, and residue suspended either in extract or in boiled extract. In the cocarboxylase synthesis experiment the liver used was from avitaminous pigeons; the O₂ uptake was measured in similar preparations of liver from normal pigeons. It will be seen that the

Table V. *Synthesis of cocarboxylase from vitamin B₁ in liver extracts, liver residue and in both combined (liver from avitaminous pigeons) and O₂ uptake of the same fractions from normal pigeon's liver*

1 hr. at 38°, pH of medium 8.5. 40 μg. vitamin B₁ HCl per g. tissue added to each sample.

No.	Sample	Cocarboxylase synthesized μg. per g. tissue	μl. O ₂ uptake per g. tissue
1	Residue in Ringer	0.25	8
2	Extract (4 ml.)	1.72	114
3	Residue in extract	5.20	1630
4	Residue in boiled extract	0.80	1825

combination of residue and extract gives both a good cocarboxylase synthesis and a high respiration. The residue alone or the extract alone give neither. The residue does not respire owing to lack of soluble substrates and coenzymes which can be supplied with boiled extract; the respiration is then restored but not the power to synthesize cocarboxylase, proving that the synthesizing enzyme was present mainly in the extract and was destroyed by boiling.¹

¹ This does not exclude the possibility that some other necessary factor might be present in the residue.

The amount of preformed cocarboxylase in normal pigeon's liver brei, if this is incubated in Ringer phosphate pH 8.5 at 28°, over a period of 4 hr., remains absolutely constant in O₂ but decreases steadily on incubation in N₂. Thus in one case the brei contained 5.4 µg. cocarboxylase per g. at the start of the experiment¹ and 5.7 µg. at the end of 4 hr. in O₂, but only 2.9 µg. per g. at the end of the same period in N₂. The decrease was 47 %. The O₂ uptake in this experiment increased linearly with time and reached 8000 µl. O₂ per g. tissue at the end of the 4 hr. period. The breakdown of cocarboxylase when O₂ is withdrawn might be partially responsible for the damaging effect of anaerobiosis on the pyruvate oxidation system of brain observed by Peters [1937, 2; cf. especially p. 11].

Effect of poisons on the synthesis of cocarboxylase

The effects of iodoacetic acid and NaF on the synthesis of cocarboxylase in liver slices are shown in Table VI. The synthesis is markedly inhibited by iodoacetate (0.0009 M) but only slightly affected by NaF (0.04 M).

Table VI. *Effect of poisons on the synthesis of cocarboxylase from vitamin B₁ in liver slices from avitaminous pigeons*

pH of medium 7.3. Incubation in O₂ at 38°, exp. 1, 30 min.; exp. 2, 50 min.

Exp. no.	Additions per g. tissue	Cocarboxylase µg. per g. tissue		Inhibition of synthesis %
		Found	Synthesized	
1	Control	1.00	—	—
	40 µg. vitamin B ₁	6.42	5.42	—
	Vitamin + NaF (0.04 M)	5.30	4.30	21
	Vitamin + IAA (0.005 M)	1.11	0.11	98
2	Control	0.61	—	—
	200 µg. vitamin B ₁	3.93	3.32	—
	Vitamin + NaF (0.04 M)	3.33	2.72	18
	Vitamin + IAA (0.0009 M)	1.63	1.02	70

Breakdown of cocarboxylase in tissues

The tissues contain a soluble enzyme (probably a phosphatase) which destroys cocarboxylase. In tissue extracts where, owing to lack of respiration, no resynthesis of cocarboxylase can take place, breakdown of preformed or

Table VII. *Cocarboxylase breakdown in tissue preparations*

Incubation at 38°. Cocarboxylase added in exps. 1, 2 and 3.

Exp. no.	Animal	Enzyme	pH	Incubation time hr.	Gas	Cocarboxylase µg. per g. tissue		%
						Before	After	
1	"Rice-fed" pigeon	Liver extract	8.5	2	O ₂	5.7	1.5	73
2	"Rice-fed" pigeon	Liver extract	7.5	1	O ₂	33.5	11.0	67
3	"Rice-fed" pigeon	Liver extract*	7.5	1	O ₂	26.4	8.5	68
4	Normal pigeon	Brain disp.†	8.5	4	N ₂	2.61	1.84	29.5
		Muscle disp.†	8.5	4	N ₂	2.63	2.23	15.1
		Liver disp.†	8.5	4	N ₂	3.84	1.22	68.0
5	Normal rabbit	Kidney cortex disp.†	8.5	4	N ₂	4.22	0.92	76.3

* In exp. 3 an aqueous liver extract was precipitated with 10 vol. acetone. The dried acetone powder was dissolved in phosphate buffer and this extract used, after centrifuging off from insoluble material.

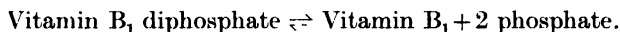
† "Disp." = dispersion.

¹ In order to secure a high liver cocarboxylase, the bird used in this experiment was injected with 3 mg. vitamin B₁ 30 min. before killing it.

added cocarboxylase occurs even in the presence of O_2 ; in minced tissues it takes place when O_2 is withdrawn. The powers of the various tissues to destroy cocarboxylase are different; liver and kidney are much more active in this respect than are skeletal muscle or brain (Table VII), thus breakdown is highest in those tissues which show the highest activity to synthesize cocarboxylase from vitamin B_1 .

DISCUSSION

The results presented in this paper would be best explained by assuming that one and the same enzyme catalyses the reaction



When energy is available (i.e. from respiration) the equilibrium is shifted towards the left. There must however be another, as yet unknown, factor which limits the extent of the synthetic reaction; this makes a further analysis of the reaction impossible and therefore the above view can only be advanced as a provisional hypothesis.

It is possible that esterification of the vitamin with inorganic phosphate is coupled with oxidation of triosephosphate,¹ the inhibition of the synthesis by iodoacetic acid is in favour of this view; but whether this would involve adenylic acid in an intermediate reaction it is impossible to decide. Failure to obtain increased synthesis with liver preparations (which are not synthesizing maximally: cf. Table I, exps. 9 and 12) on addition of adenosine triphosphate, phosphoglyceric acid, or both together, is evidence against a direct transfer of phosphate from either compound.

In yeast Lipschitz *et al.* [1938] think that phosphorylation of the vitamin may be coupled with triosephosphate oxidation. Through the kindness of Dr Lipschitz I have been able to test the ability of their yeast to synthesize cocarboxylase from vitamin B_1 after alkaline washing (unpublished experiments), and found that synthesis does occur but only to a small extent, not more than with liver preparations. In the presence of pyruvate, synthesis is dependent upon the presence of cozymase but the presence of hexosediphosphate is not essential. These points obviously require further investigation.

The physiological significance of the higher synthetic activity of liver (and kidney) as compared with other tissues, and the possible conveyance of vitamin B_1 as such in the blood, have been discussed in a previous paper [Banga *et al.* 1939; cf. Goodhart & Sinclair, 1939].

SUMMARY

1. Liver slices, brei or "dispersions" from avitaminous pigeons convert added vitamin B_1 into cocarboxylase to an extent which does not greatly surpass the normal cocarboxylase content of the tissue. Optimally, with small amounts of vitamin, 30 % of this may be converted into cocarboxylase in 30–60 min. at 38° .

2. Brain and muscle preparations are much less active; preparations from duodenal mucosa (pig) showed no activity under various experimental conditions.

3. With liver, synthesis is dependent on an active respiration and is highest at alkaline reactions (optimum about pH 8.5). The synthesis is inhibited by iodoacetic acid but not much affected by NaF.

4. An essential part of the enzyme catalysing the synthesis is soluble.

¹ The fact that synthesis with liver is much reduced in N_2 may be due to the low glycolytic activity of liver preparations.

5. Soluble enzymes which, when no respiration is possible, destroy cocarboxylase, are present in liver, kidney, muscle and brain; their activity is highest in those tissues (liver and kidney) which also show the highest synthetic capacity.

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CLVI. THE BASIC AMINO-ACIDS OF LEAF PROTEINS. WITH A DISCUSSION OF VARIOUS METHODS OF ANALYSIS

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DURING the past few years numerous protein preparations have been made in this laboratory from forage plants at various stages of growth and growing under different climatic and manurial conditions. To compare these products one with another, and with the whole leaf protein of which they represent a part, it has been necessary to make amino-acid analyses not only of the protein preparations themselves, which may contain some 13–15% N, but also of leaf residues containing unextracted protein or of leaf material containing the whole of the protein, both of which latter products, after removal of non-protein-N, are rich in carbohydrate and may contain only 3 or 4% N. It has been thought advisable, therefore, to find out first of all whether the amino-acids set free during acid hydrolysis of the protein would condense with products derived from the carbohydrate and thus give low yields on subsequent analysis.

Miller [1935] has already carried out exploratory work on these lines with respect to the bases, and his conclusions can be summarized as follows.

(1) The large-scale method of Vickery and his co-workers [cf. Vickery & Shore, 1932] was the most reliable, but the amount of material required (50 g. or more) made its general use impracticable.

(2) Block's [1934, 2] small-scale adaptation of the above method, after certain modifications suggested by his (Miller's) experience, gave values which agreed closely with those obtained by the large-scale procedure.

(3) The N-distribution method of Van Slyke gave values with leaf proteins which could only be regarded as extravagant.

Miller also expressed the opinion that the destruction of bases which might occur during acid hydrolysis of leaf proteins in the presence of considerable amounts of carbohydrate impurities would not be very profound, even though the earlier work of Roxas [1916] had shown that small amounts of humin-N were formed when the free bases were heated with carbohydrates in acid solution. But the recent work in this laboratory of Bailey [1937] and of Lugg [see Chibnall, 1939, Appendix I] has made it clear that the impurities present in leaf products are those (e.g. mucilage, pentosan) which give rise to much furfuraldehyde on acid hydrolysis, and that under these conditions considerable destruction of certain of the amino-acids does indeed occur. Bailey, for instance, has shown that, in the case of cysteine, destruction or modification occurs more readily at the time of its actual liberation from the protein molecule than when it is present as the free amino-acid, while Lugg [1938, 1, 2] has pointed out the difficulties which attend the estimation of cystine, methionine, tyrosine and tryptophan in protein products containing such impurities. These findings have necessitated a re-examination of the methods of base analysis to ascertain what, if any, loss of each individual base occurs during hydrolysis under similar conditions.

As the amount of material available was in most cases limited the method chosen for investigation was that of Block [1934, 2], whose original procedure has been modified by him in recent years as his own extensive experience has suggested [cf. Block, 1938]. In the course of the present work it has likewise been found that certain suitable though minor changes can be recommended, and the procedure finally adopted for the analysis of leaf products, which incorporates suggestions made by Miller [1935] and by Ayre [1938], has been rigorously tested against appropriate mixtures of bases as well as against certain standard proteins which had already been analysed by the large-scale methods of Vickery and his co-workers. It will facilitate a critical discussion of the results obtained if the new procedure be first of all described in detail.

The modified method of base analysis

All concentrations are carried out *in vacuo* at 45° and all precipitates are washed three times unless otherwise stated.

Hydrolysis. 2.5–3.0 g. of protein are hydrolysed by boiling under reflux with a mixture of 5.5 ml. H_2SO_4 and 19.5 ml. water for 18–22 hr. (It is necessary to heat on the water bath until the protein has dissolved.) The hydrolysate is cooled, transferred to a 250 ml. pyrex centrifuge cup and treated with hot saturated baryta to pH 3 (indicator thymol blue). (Block [1934, 2] advised pH 3–6 but if the solution is made less acid than pH 3 the complete extraction of N from the BaSO_4 precipitate becomes difficult if the hydrolysis has given much acid humin.)

The precipitate is centrifuged, transferred to an end-runner mill (a heavy mortar and pestle will suffice) and ground with acidulated water (pH 3). The resulting cream is then poured into a beaker, boiled with vigorous stirring for 10 min., cooled and again centrifuged. The washing is twice repeated, about 400 ml. water being used each time. The solution and washings are concentrated to 20 ml. and transferred to a 250 ml. centrifuge cup, any slight precipitate of BaSO_4 being ignored.

Histidine fraction. Ag_2O and H_2SO_4 (sufficient to maintain the pH) are added until Ag_2SO_4 crystallizes in appreciable amount; AgNO_3 is then added to ensure excess Ag ion (brown precipitate with baryta). This variation of Block's procedure is recommended, as the exclusive use of AgNO_3 introduces nitrate ions in such concentration that partial destruction of lysine may occur at a later stage. The solution is next adjusted to pH 7.2–7.4 (blue-green with bromothymol blue) by the addition of cold saturated baryta; it is important that during this operation the stirring be efficient since a local concentration of baryta tends to precipitate arginine silver, which does not redissolve very readily above pH 4. If the end-point is exceeded (so that the solution is alkaline to phenolphthalein) the solution must be acidified to pH 4.0 (Congo red), stirred for several minutes and the adjustment of pH repeated. The precipitate of histidine silver is then centrifuged and washed twice with water (50–60 ml. in all), the clear liquid and washings (A) being acidified to pH 5 and treated for arginine as described below. The histidine precipitate is suspended in water acidified with H_2SO_4 to pH 4 (bromocresol green) and freed from silver by H_2S under pressure. The end-point is invariably marked by coagulation of the Ag_2S . The precipitate of Ag_2S and BaSO_4 is centrifuged, thoroughly washed and the collected liquors concentrated to 5 ml. The concentrate is transferred to a 100 ml. centrifuge bottle (final volume 15 ml.), 50 ml. of a saturated solution of HgSO_4 in 5% H_2SO_4 are added and the mixture placed in the ice box for at least 12 hr. (It is very convenient to leave histidine in this form until the arginine and lysine have been dealt with.)

The mercury compound is centrifuged, washed once with the reagent and decomposed with H_2S . The filtrate and washings are concentrated to 5 ml., neutralized with baryta, BaCO_3 added and the mixture brought to the boil. $\text{Cu}(\text{OH})_2$ or CuCO_3 is now added until in excess and the boiling maintained for 15 min. The mixture is cooled for at least 30 min. at 0° and the precipitate removed and washed. The filtrate is acidified until the solution becomes pale green, Cu is removed with H_2S and the CuS washed. The filtrate is now concentrated to 50 ml., an aliquot withdrawn for determination of total N and the remainder concentrated to 2 ml. Solid flavianic acid (15 mg. per mg. N) is added and the histidine diflavianate allowed to crystallize for 2-3 days at 0° . The diflavianate is filtered, washed with a little cold water containing flavianic acid, with ice-cold absolute alcohol, then with ether and finally dried at 105° . If the canary-yellow solid turns orange it must be purified by recrystallization. The S content should be 8.17 %, but in keeping with the experience of Vickery and his colleagues the products isolated in this laboratory have generally given a slightly higher value, *circa* 8.38 % (Schoeller, micro-Carius). The factor for converting the weight of diflavianate into histidine is 0.198.

Arginine fraction. The solution (A) is concentrated, in the presence of any precipitate which may have formed, to 25 ml., transferred to a 250 ml. centrifuge cup, tested for excess silver and the pH adjusted to 12-13 with a hot saturated solution of baryta (lanacyl blue or alizarin yellow). The precipitate is centrifuged, washed twice with cold saturated baryta and the total volume of liquid noted for subsequent correction for the solubility of arginine silver. The liquid (L) is treated later for lysine.

The precipitate is suspended in water, H_2SO_4 is added to pH 4, the silver removed with H_2S under pressure (coagulation is again a test) and the precipitate washed. The solution and washings are concentrated to 100 ml.: 1 ml. is withdrawn for determination of total N, the remainder adjusted to pH 5-6 (bromocresol green) and concentrated. Any precipitate of BaSO_4 is removed by filtering through a No. 42 Whatman filter paper and the final solution is concentrated to 2 ml. in a 150 ml. flask. The capillary and Claisen head of the flask are washed down to make a final volume of 10 ml.

The solution is heated on the water bath at 100° , the required amount of flavianic acid added in concentrated aqueous solution (6 mg. acid per mg. N) and the mixture shaken until crystallization commences. After standing overnight at 0° the flavianate is filtered, washed successively with cold water, alcohol and ether, dried at 105° and weighed. The S content should be 6.56 %, but the products isolated in this laboratory have always given a slightly higher value, *circa* 6.72 % (Schoeller, micro-Carius). The factor for converting arginine flavianate into arginine is 0.3566.

Lysine fraction. Use is made of the finding of Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether, thus eliminating the removal of NH_3 with NaOH .

The filtrate (L) is acidified to pH 6; 1.1 ml. conc. H_2SO_4 are added and excess silver removed as Ag_2S . The precipitate is well washed and the filtrate concentrated to 15 ml. Care should be taken not to reduce the volume below this owing to the presence of HNO_3 .

The solution is transferred to a centrifuge cup (volume now 30 ml.) and heated to 90° on a water bath for 3 min.: 50 ml. of a 20 % solution of 24-phosphotungstic acid (A.R.) in 5 % H_2SO_4 , also at 90° , are added and the phosphotungstate allowed to crystallize overnight at $15-20^\circ$. The precipitate is washed twice with a solution of 2.5 % phosphotungstic acid in 5 % H_2SO_4 . The phosphotungstates are

then decomposed by disintegrating with 50 ml. of water (to which has been added 1 ml. H_2SO_4) and a liberal quantity of amyl alcohol-ether (25 parts ether, 20 parts amyl alcohol, 1 part ethyl alcohol) and the mixture centrifuged. The clear liquids are poured into a separating funnel. The residue, which consists of ammonium phosphotungstate, is washed twice, as before, using smaller quantities of acidified water and organic solvents, the washings being added to the mixed liquids in the separating funnel. After allowing the solutions to clear, the aqueous solution is drawn off and shaken out three times successively with amyl alcohol-ether. The combined organic solutions are shaken once with a small quantity of acidified water, and the latter solution washed once with amyl alcohol-ether. The aqueous solutions are then combined and given a final washing with a small quantity of amyl alcohol-ether.

This aqueous solution is then concentrated to 100 ml.; aliquots are withdrawn for determination of total N and the remainder treated for the separation of lysine picrate. The solution is adjusted to pH 6.5-7.0 (bromothymol blue) with baryta; 1.0 g. BaCO_3 is added, the precipitate is centrifuged and well washed. The filtrate is then concentrated in the presence of BaCO_3 (1.0 g.) and filtered through a No. 42 filter paper. The final solution is concentrated to 1 ml., the capillary and flask head washed down with 6 ml. 93% alcohol, the resulting liquid is adjusted with absolute alcohol or water so that a faint turbidity remains and is then cooled to 0° . An alcoholic solution of picric acid (8.1 mg. acid per mg. N), cooled to 0° , is then added. (It is necessary to cool to 0° because the use of a warm alcoholic solution of picric acid may lead to the formation of an oil.)

The picrate is allowed to crystallize overnight at 0° , washed with ice-cold alcohol and ether, dried at 105° and weighed (in case loss occurs during recrystallization); it is recrystallized from water at 0° (solubility 3.4 mg. per ml. at 0°), dried as before and weighed. It has been the unanimous experience of those who have used the original Block or the above-mentioned modified procedure in this laboratory that the lysine picrate, prepared by using either A.R. picric acid or the product purified according to the directions of Block [1938], will not explode above 260° unless recrystallized. The factor for the conversion of the weight of lysine picrate into lysine is 0.39.

Corrections for solubilities and overall losses in procedure

An arginine silver correction of 1.1 mg. N per 100 ml. [Gulewitsch, 1899] has been applied by Vickery & Shore [1932] and by Vickery & White [1933] in their large-scale analyses of ovalbumin and casein respectively. A perusal of Block's earlier publications suggests that he also, in certain instances, has applied this correction, but no specific reference to it is made in the paper [1934, 2] which describes his original procedure, while in his later review [1938] he merely quotes, in a special table, without further comment, the solubility value, together with those of other base salts. It is to be remembered, of course, that the solubility of a salt such as arginine silver in any given solution will be governed by the amounts of other amino-acids or their salts present [cf. Cohn, 1935], a point that is strikingly illustrated by the data concerning the solubility of arginine phosphotungstate discussed in a later section; consequently the employment of such a correction in the case of protein hydrolysates is somewhat arbitrary and opportunity has been taken to investigate the matter, in admittedly simpler systems, as part of an enquiry into the losses inherent in the above-mentioned procedure for separating the three bases.

For this purpose arginine and histidine were prepared from their recrystallized flavianates and lysine from its recrystallized picrate, the amount of each

base taken in the experiments about to be described being equivalent to the respective occurrence in 2.5 g. of a typical leaf protein. In exp. 1 arginine alone was used, silver being precipitated at pH 7 as though histidine were present. 1.1 mg. of N were lost in this "histidine precipitate". After precipitation of the arginine silver at pH 14 the mother liquor contained 3.3 mg. of N, equivalent to a solubility of 2.0 mg. per 100 ml. Two other experiments (unrecorded) gave similar results. In exps. 2 and 3, with histidine also present, the histidine silver precipitates contained 2.2 and 1.2 mg. of N respectively in excess of the histidine-N present in the original solution, while the arginine silver mother liquors contained 1.4 mg. and 3.3 mg. of N, equivalent to solubilities of 1.0 mg. and 1.4 mg. per 100 ml. respectively. In exps. 4 and 5, containing all three of

Table 1. *Analysis of various mixtures of bases*

Arginine-N						
Corrected for arginine silver 1.1 mg. N per 100 ml.						
	Taken mg.	Recovered mg.	Loss mg.	Recovered		Loss mg.
				mg.	%	
1	37.9	33.4	4.5	36.1	95.2	1.8
2	37.9	34.3	3.6	35.9	94.7	2.0
3	37.9	33.4	4.5	35.2	92.9	2.7
4	38.2	33.7	4.5	35.9	94.0	2.3
5	38.2	33.1	5.1	35.3	92.4	2.9
6	42.8	38.8	4.0	40.5	94.5	2.3

Histidine-N					Lysine-N			
	Taken mg.	Recovered		Loss mg.	Taken mg.	Recovered		Loss mg.
		mg.	%			mg.	%	
1	—	—	—	—	—	—	—	—
2	9.0	—	—	—	—	—	—	—
3	9.0	—	—	—	—	—	—	—
4	6.4	6.2	96.8	0.2	18.4	16.9	91.8	1.5
5	6.4	6.0	93.8	0.4	18.4	17.6	95.5	0.8
6	10.2	10.0	98.5	0.2	25.9	24.1	93.0	1.8

the bases, the arginine silver mother liquors contained 3.0 mg. and 3.6 mg. in excess of the known content of lysine-N, equivalent to solubilities of 1.5 mg. and 1.8 mg. per 100 ml. respectively. Exp. 6 was similar to 4 and 5, except that the mixture was submitted to the usual acid hydrolysis before separation, the solubility of the arginine silver in this case being 1.6 mg. per 100 ml. In all these experiments, as in the protein analyses quoted later, there was no loss on recrystallization of the arginine flavianate. These results show that under the working conditions of these small-scale experiments the apparent solubility of arginine silver may be a little greater than that recorded by Gulewitsch [1899], and that it is not appreciably increased in the presence of lysine. Assuming for convenience, therefore, that the Gulewitsch correction (1.1 mg. N per 100 ml.) holds, the overall losses in the estimation of arginine, of mean value 2.6 mg. N, are to be ascribed to possible underestimation of this solubility factor and to precipitation with the histidine silver at pH 7. Whether such an overall correction is valid in the case of a protein hydrolysate must remain a matter of conjecture until new methods of analysis are found which permit a direct

determination of solubility under such conditions [cf. Bergmann & Stein, 1939] and it is only with this reservation that the correction has been applied in the analyses quoted later in Tables II and V.

The estimation of N in the histidine silver fraction of expts. 2-7 confirms the findings of Vickery that this salt is completely insoluble and that the mean overall loss of 0.3 mg. is due to the difficulty of isolating such small amounts of histidine diflavinate. With the reservation given above, correction for this small overall loss has been applied in the analyses quoted later in Tables II and V. In this connexion certain inconsistencies in Block's publications must be noted. A histidine value of 0.91 % for eight mammalian serum proteins quoted by Block *et al.* [1934] was corrected to 2.1 % by Block [1934, 1] for "the average loss of histidine incident to the method of estimation", but in his later review [1938] he states that the "recovery of histidine as diflavinate is approximately 75 % of that present". Such statements are entirely unsupported by any published experimental evidence and in view of the results quoted in Tables I and III of the present paper they appear to be unjustified.

The average overall loss of 1.6 mg. of lysine-N in expts. 4-11 (Tables I and III) covers the solubility of lysine phosphotungstate (volume about 175 ml.) and all the incidental losses of procedure. As will be discussed later, there is clear evidence that in certain protein hydrolysates the solubility of arginine phosphotungstate is no greater than that determined by Van Slyke in an equivalent mineral acid solution, while in others this value is greatly exceeded, so that in the case of the protein analyses quoted below the true overall loss of lysine-N may well be greater (or less) than the value found for the simple base mixture. This reservation, of course, applies with equal force to the large-scale work of Vickery and co-workers and also to the Van Slyke method of base analysis, consequently it cannot be regarded as one of the contributory causes of the high values invariably obtained for lysine by the latter procedure. To clarify the situation we must again await the development of methods for the estimation of this base in which the residual solubility can be directly computed: meanwhile an overall correction of 1.6 mg. of lysine-N has been applied in the protein analyses given below.

Analysis of certain standard proteins

The ovalbumin and casein I were kindly supplied by Dr Vickery, being samples of the actual preparations analysed by Vickery & Shore [1932] and Vickery & White [1933] respectively; the edestin and the other samples of casein were prepared in this laboratory and contained 18.6, 14.83 and 15.7 % N respectively. The results of analysis, together with those of other workers for comparison, are collected in Table II.

It will be seen that the values for the individual bases, when calculated simply on the weights of crystalline derivatives, tend, in the small-scale procedures, to be lower than those obtained by working on a larger scale. This is to be expected, since in the latter case the mother liquors from the separation of the main crop of derivate can be subsequently worked over to increase the yield. As has been shown earlier, this drawback to the use of the small-scale procedure can, with certain assumptions, be overcome by the use of overall corrections for losses, and when these are applied the agreement between the two sets of results is as close as one could reasonably expect.

Table II. *Base analysis of standard proteins*

(Values quoted are percentages of total protein-N)

Protein	Analysed by	Arginine-N			Histidine-N		Lysine-N	
		Wt. of protein used g.	Found (corrected for arginine silver 1.1 mg. N per 100 ml.)	Corrected for overall losses (+ 2.6 mg. N)	Found	Corrected for overall losses (+ 0.3 mg. N)	Found	Corrected for overall losses (+ 1.6 mg. N)
Ovalbumin	Author	2.89	11.05	11.65	2.55	2.63	5.5	5.85
Ovalbumin	Author	2.52	11.6	12.3	(Lost)	—	5.6	6.00
Ovalbumin	Vickery & Shore [1932]	65.0	11.6	—	2.1	—	5.6	—
Ovalbumin	Vickery & Shore [1932]	65.0	10.7	—	2.6	—	6.1	—
Casein I	Author	2.7	7.7	8.3	3.02	3.1	7.4	7.8
Casein II	Author	2.3	7.5	7.8	—	—	—	—
Casein III	Author	2.61	7.8	8.4	3.2	3.3	7.45	7.85
Casein I	Vickery & White [1933]	100	7.7	—	—	—	—	—
Casein I	Vickery & White [1933]	100	8.2	—	3.2	—	7.72	—
Edestin	Author	2.56	26.4	27.0	3.02	3.08	2.25	2.59
Edestin	Author	2.46	26.2	26.7	2.84	2.89	2.00	2.35
Edestin	Vickery & Leavenworth [1928]	366	27.4	—	3.04	—	2.26	—
Edestin	Vickery & Leavenworth [1928]	50	23.8	—	2.6	—	2.04	—
	Block [1934, 2]	2.5	27.05*	—	3.06	—	2.17	—

* No mention is made of any correction for arginine silver.

General criticism of methods of base analysis

From the experience gained in the development of the present research the following criticisms may be levelled against the small-scale gravimetric method of analysis. (1) The use of AgNO_3 , even in the limited amounts suggested in the procedure detailed above, may cause loss of base, particularly of lysine. It was found impossible, however, under the conditions of these experiments, to introduce an excess of Ag ions solely by means of Ag_2O or Ag_2SO_4 . (2) If the lysine fraction is relatively impure, and this occurred occasionally in the analyses of leaf proteins, the lysine dipicrate may separate in the form of an oil from which it was found impossible to regenerate the base quantitatively for reprecipitation. Repetition of the whole base analysis was therefore necessary in such cases. (3) The estimation of histidine is the least satisfactory of the three bases, as Vickery and his co-workers found when working on a large scale. This is due in part to the small amount of the amino-acid present in most protein hydrolysates, and in part to the difficulty of obtaining the diflavanate in a reasonable state of purity. If, for instance, a large excess of flavianic acid be added to prevent the formation of a monoflavanate, the excess acid may crystallize out and as this must subsequently be washed away with cold water a loss of diflavanate may result. It was presumably to overcome some of these difficulties that Block has recently suggested the use of nitranilic acid as a precipitant for this base, but he has not as yet given any data for the recovery of histidine nitranilate from pure base solutions or from protein hydrolysates. Finally, from what has been said above concerning the possible vicarious

solubilities of arginine silver and lysine phosphotungstate, it may well be that both the large- and small-scale gravimetric methods are still underestimating the content of one or more of the bases in certain proteins.

This latter statement in no way implies, however, that the higher values usually obtained for histidine and lysine by the indirect Van Slyke method of base analysis are valid. Much has been written in the past as to the trustworthiness of this method, and some of the criticisms put forward have been recently summarized by Calvery [1938], who points out that the procedure has frequently been used in a way not intended by Van Slyke, namely, as an absolute rather than as a comparative method of analysis. The main objections are: (1) The phosphotungstate precipitate rarely, if ever, contains exclusively bases; small amounts of tyrosine, tryptophan and phenylalanine have been isolated from it in certain cases while Gortner & Sandström [1925] have pointed out the possible interference of proline. (2) If large amounts of cystine are present in the protein hydrolysates the determination of arginine is affected, since concentrated NaOH converts up to 20 % of the cystine-N into NH_3 . (3) Enhanced values for arginine-N and amino-N are reflected in the calculated values for lysine-N and histidine-N.

Another important observation has, however, been overlooked by Calvery, namely, that the solubility of arginine phosphotungstate in a protein hydrolysate may be greater than the value (about 1.6 mg. N per 100 ml.) recorded by Van Slyke. This was first pointed out by Vickery & Leavenworth [1928], who were able to isolate from the phosphotungstate filtrate of edestin, *via* the silver salt, more than twice the expected amount of arginine flavianate. In the course of the present work a similar result was given by myosin, yet in the case of casein, and also of many leaf proteins, the Van Slyke solubility factor was valid, since the amount of arginine flavianate obtained from the phosphotungstate precipitate, when given this correction, was equal to that obtained from the hydrolysate direct. Clearly the solubility of arginine phosphotungstate depends on the amino-acid mixture present in the protein hydrolysate [cf. Cohn, 1935] and the fact that this may be in excess of the Van Slyke value lends added weight to the remarks which follow.

Miller [1935], working in this laboratory, applied both the gravimetric and indirect methods of analysis to cocksfoot leaf protein, and found that the total N precipitated by phosphotungstic acid under the Van Slyke conditions was greatly in excess of the total basic N determined gravimetrically by the large-scale procedure of Vickery, the latter value being more or less equivalent to the total N precipitated by phosphotungstic acid under the Hausmann conditions. Since in the Van Slyke procedure the precipitation with phosphotungstic acid is carried out in the presence of HCl—instead of H_2SO_4 as in the Hausmann procedure—he suggested that the high value obtained in the former case might be due to precipitation of certain non-basic substances under such conditions. Further consideration, however, does not lend support to such a view, for these differences can be accounted for in large part in terms of the solubility of arginine phosphotungstate, as recorded by Van Slyke, and of the different volumes of solution from which the precipitations are made in the two cases. Indeed such relatively large volumes of solution are employed in the Hausmann procedure that the phosphotungstate filtrate and washings must contain much arginine-N, and the general agreement between the Hausmann basic-N and the gravimetric total basic N, which was stressed by Osborne [1924, p. 73], must be largely fortuitous and due to the presence of non-basic products in the Hausmann as well as in the Van Slyke precipitate. In view of all these criticisms it is

impossible to agree with the conclusion of Calvery [1938] that "in comparing the Van Slyke method with that of Kossel (i.e. Vickery, Block), it is generally true that in the case of lysine the Van Slyke method is more accurate".

Analysis of a mixture of the three bases after hydrolysis in the presence of various carbohydrates

Before applying the above-mentioned gravimetric procedure to the analysis of leaf protein preparations it was necessary to ascertain the effect, if any, which the carbohydrates known to be associated with the proteins in these products would have on the recovery of the three individual bases. Appropriate mixtures of the bases (representing 2.5 g. of a leaf protein) were accordingly hydrolysed in the presence of various carbohydrates which yield much furfuraldehyde under such conditions, and the subsequent analyses are given in Table III, the value for recovered arginine-N carrying a silver correction of 1.1 mg. per 100 ml. In agreement with the previous finding of Roxas [1916] arginine has suffered a loss which is roughly proportional to the amount of carbohydrate present, but the change undergone by the amino-acid has not been ascertained, save that no NH_3 is produced and that the humin-N formed accounts for only a part of the lost arginine-N. Histidine recoveries are irregular, and, except in the case of exp. 15, are probably associated with the difficulties of estimation (see earlier) rather than with destruction of the amino-acid. Lysine recoveries in exps. 8-14 are normal, but the small loss shown in exp. 15 may be significant, since it will be shown in a later paper that much destruction of this amino-acid can occur if the amount of impurity be increased to the equivalent of 10 times the protein present. That destruction of base, particularly of arginine, may be more extensive at the time of liberation either as peptide or as free amino-acid during hydrolysis of a protein [cf. Bailey, 1937] follows from the results of exps. 12 and 13, although in these particular cases the total amount of arginine concerned was very much greater than in the others, wherein the arginine content was designedly made more or less equivalent to that of a typical leaf protein, and comparison may not be valid. In view of these relatively large losses of arginine in the presence of much carbohydrate and the uncertainty in the recovery of histidine it has been thought advisable in dealing with what can only be regarded as grossly impure protein preparations, e.g. those with 8% N or less (see section on whole grass analyses) to limit the base analysis to the determination of lysine.

Table III. *Recovery of bases after hydrolysis in the presence of various carbohydrates*

Exp.	Added carbohydrate	Wt. g.	Arginine-N				Histidine-N				Lysine-N			
			Taken mg.	Recovered			Taken mg.	Recovered			Taken mg.	Recovered		
				mg.	%			mg.	%			mg.	%	
8	—	—	42.8	40.4	94.5		10.2	10.05	98.5		25.9	24.1	93	
9	Arabinose	0.25	42.8	37.0	86.5		10.2	8.1	79.5		25.9	24.6	95	
10	Arabinose (a)	0.25	42.8	36.6	85.5		10.2	9.9	97		25.9	23.9	92.4	
11	Arabinose	0.5	52.0	43.7	84.0		7.1	7.1	100		28.4	26.4	93	
12	Arabinose (b)	0.5	136.3	114.5	84.0		15.1	14.1	93.5		11.3	10.8	96	
13	None (b)	—	136.3	131.0	96.0		15.1	14.6	96.8		11.3	10.6	93.0	
14	Xylose	0.3	52.0	45.5	87.6		7.1	7.02	98.7		28.4	27.0	95	
15	Hemicellulose	2.5	52.0	37.8	72.7		7.1	4.9	69		28.4	25.5	90	

(a) 0.1 g. cystine and 0.1 g. tryptophan were added to this mixture before hydrolysis.

(b) 2.68 g. edestin instead of mixed bases were used in this experiment. The amount of each base was calculated from the data of Table II, i.e. arginine-N = 27.4%; histidine-N = 3.04%; lysine-N = 2.26%.

Base analysis of various leaf protein preparations

The samples of forage proteins used had been prepared by Chibnall and his colleagues in past years by the "used ether-water" method and most of them have been recently analysed by Lugg [1938, 1, 2] for amide-N, tyrosine-N, tryptophan-N, cystine-N and methionine-N. For continuity the series numbers quoted by Lugg have been retained in Table V. The samples of maize and castor bean proteins were those prepared by Chibnall, using the "ether" method [cf. Chibnall, 1924; Chibnall & Nolan, 1924]. All these products contained 12.5–15.7 % N and since there is reason to believe that pure leaf proteins should contain about 17 % N [cf. Chibnall, 1939] it has been assumed that the adulterant—of the pentosan nature referred to above—will cause destruction of arginine during acid hydrolysis in amount proportional to that of the adulterant present (cf. Table III).

Table IV. *Factors used for correcting values of arginine-N in leaf protein preparations*

% N	17.0	16.0	15.0	14.5	12.5–13.0
Calculated % adulterant	0	5.9	11.8	14.7	20.0–30.0
Factor for loss	1.06	1.10	1.15	1.17	1.20

Table V. *Base analysis of leaf protein preparations*

(Figures given are in percentages of total protein-N)

Preparation no.	Source	Date	Total N (ash-free) %	Arginine-N	Histidine-N	Lysine-N
4	<i>Dactylis glomerata</i> (cocksfoot grass)	29. ix. 36	14.25	13.9	2.3	6.3; 6.1
2E(b)	"	7. vi. 37	12.76	15.5; 15.2	2.4; 2.6	6.0
16	<i>Lolium Italicum</i> (Italian rye-grass)	26. vi. 33	14.1	13.4	2.4	5.9
14	<i>Lolium perenne</i> (perennial rye-grass)	6. xi. 33	13.0	14.5	2.4	6.2
3E(a)	"	9. vi. 37	13.75	13.4	—	5.8
18	<i>Poa trivialis</i> (rough-stalked meadow grass)	27. vi. 33	14.1	16.4	2.3	5.6
—	"	4. x. 32	13.6	15.3	2.1	5.4
20	<i>Festuca rubra</i> var. Fallax (chewing fescue)	29. vi. 33	14.7	14.3	1.9	5.2
19	"	26. ix. 32	14.4	14.6	2.0	5.7
22	<i>Cynosurus cristatus</i> (crested dog's tail)	12. vi. 33	14.3	15.1	2.6	5.8
25	<i>Medicago sativa</i> (lucerne)	29. vi. 33	14.4	15.1	2.3	7.0
26	<i>Trifolium repens</i> (wild white clover)	18. x. 33	13.3	15.4	1.6	6.6
27	<i>Trifolium pratense</i> (red clover)	23. x. 33	12.95	14.9	2.3	6.5
32	<i>Spinacea oleracea</i> (spinach)	18. vi. 24	15.4	14.1	2.2	6.2
—	✓ <i>Beta cicla</i> (beet spinach)	1. xi. 37	15.0	12.4	2.1	6.8 ✓ —
5E1	"	3. xii. 37	14.2	13.1	2.4	6.6
—	<i>Zea Mays</i> (maize)	1924	13.95	14.4	2.1	6.1
—	<i>Ricinus communis</i> (castor bean)	1924	12.55	12.9	2.2	6.5

The factors used in correcting for this loss are given in Table IV, and in the light of the previous discussion of expts. 12 and 13 they can only be regarded as minimal, a point that must be remembered in comparing the values for arginine-N given in Table V. Factors of 1.07 and 1.03 for lysine-N and histidine-N respectively have also been applied, irrespective of the amount of adulterant present.

The base analyses, like those of Lugg for the other amino-acids and the amide-N, exhibit surprisingly little variation from species to species, showing that leaf proteins, as a class, are of much more uniform composition than, for instance, a typical class of seed proteins such as the globulins [cf. Osborne, 1924]. The importance of this observation in problems connected with agricultural nutrition has already been discussed at some length by Chibnall [1939].

Base analysis of the whole protein of leaves

The extraction of protein material from leaves by the methods used in preparing the samples mentioned in Table V depends on the efficacy of the grinding operations employed, and the debris of leaf material may contain 70–85% of the total amount of protein originally present in the untreated leaves. From a nutritive point of view the composition of this unextracted protein is of equal importance to that of the isolated product, but the difficulties attending its analysis are great, on account of the large amount of non-protein material with which it is associated. These leaf residues, or, in certain cases, the fresh leaves containing the whole of the protein, were prepared for analysis in the following way.

The dried and ground leaf material was first treated at boiling point with 0.01 *M* acetic acid to remove all soluble non-protein nitrogenous products, sugars etc., then successively with graded strengths of boiling alcohol to remove most of the lipin, with boiling 0.005 *M* citric acid (which usually reduces the ultimate ash content but removes only a negligible amount of nitrogenous material), twice with boiling alcohol (or until extract was almost free from chlorophyll) and finally with warm ether. Such residues, regarded as proteins, are grossly impure, since they will contain only from 2% N to (rarely) 8% N. In one particular case the dried grass contained 4.2% N, and after treatment in this way the leaf residue contained 5.6% N. If this N represents protein, and this is assumed, then, from the point of view of hydrolysis, the impurity present was about 200% and to reduce this the leaf residue was boiled for 24 hr. with 7.5% H_2SO_4 in amount such that, on filtration and concentration of the filtrate, the correct conditions for a base analysis could be achieved. About 92% of the residual leaf-N was extracted in this way and, by difference, the extracted material contained 7.9%, so that the subsequent hydrolysis took place in the presence of 100% impurity. Reference to Table III shows that both arginine and histidine may suffer considerable destruction under such conditions and lysine was regarded as the only base which could be estimated with any reliability, the recovery being taken as 90%. In another case the residual leaf material already contained 8% N, and opportunity was taken to compare the lysine values both with and without this preliminary extraction with dilute H_2SO_4 .

The results, together with those of corresponding protein preparations, are given in Table VI, and they show that within the limits to which we can work at the present time the values for lysine, like those for amide-N and the other amino-acids recorded by Lugg [1939; cf. Chibnall, 1939] exhibit but little

Table VI. *Lysine analyses of various protein fractions of leaves*

Preparation no.	Source	Date	Season	Material*	% of total leaf protein represented	Total N %	Lysine-N % of total protein-N
2Eb	<i>Dactylis glomerata</i> (cocksfoot)	7. vi. 37	Spring	E	25	12.8	6.0
—	"	5. vi. 32	"	W	90	8.0†	5.9
—	"	5. vi. 32	"	W	100	8.0†	6.1
—	"	29. ix. 36	Autumn	E	39.4	14.2	6.2
1W	"	29. ix. 36	"	W	91	7.5†	5.9
3E (a)	<i>Lolium perenne</i> (perennial rye-grass)	9. vi. 37	Spring	E	24	13.8	5.8
—	"	6. xi. 33	Autumn	E	21.8	13.0	6.2
4W	"	29. xi. 37	"	W	89.3	5.6†	5.9
4R	"	29. xi. 37	"	R	56	3.4†	5.6
—	<i>Beta cicla</i> (beet spinach)	6. xi. 37	"	E	—	15.1	6.8
—	"	6. xi. 37	"	W	92.5	6.7†	6.7
5R	"	3. xii. 37	"	R	52	4.1†	6.6
5E	"	3. xii. 37	"	E	—	14.2	6.6

* Extracted protein (E); protein of leaf residues (R); whole protein of leaves (W).

† N content of leaf material before preliminary extraction with dilute H_2SO_4 .

‡ No preliminary extraction with dilute H_2SO_4 .

variation as between isolated protein, extracted residue and whole protein of leaf. Moreover, there is but little variation within a single species with season. The implications of these findings have been discussed in some detail by Lugg and Chibnall in the communications cited above.

SUMMARY

Block's [1934] small-scale adaptation of the Vickery method for the estimation of the basic amino-acids of proteins has been investigated in detail. Certain minor modifications are recommended, and the modified procedure has been tested against appropriate mixtures of bases to obtain some idea of the corrections to be applied for arginine silver and overall losses.

Various standard proteins have been analysed by this modified procedure and, after correction, the results compare favourably with those obtained by Vickery and his co-workers.

A general criticism has been made of certain direct and indirect methods of base analysis.

Hydrolysis of the three individual bases in the presence of various furfuraldehyde-yielding carbohydrates has shown that arginine suffers a loss which is roughly proportional to the amount of carbohydrate present, that histidine recoveries may be irregular and that lysine recoveries are normal unless a large excess of carbohydrate is present.

In the light of these results it has been possible to obtain fairly satisfactory analyses for all three bases of leaf protein preparations containing from 12 to 16 % N.

Leaf material relatively poor in protein (containing less than 8 % protein-N) requires special preliminary treatment, and it is considered that the only base which can be estimated with any reliability is lysine.

In keeping with the previous analyses for certain other amino-acids and the amide-N by Lugg, the base analyses of leaf proteins exhibit but little variation from species to species and within a single species with season.

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CLVII. PROTEINS AND OTHER NITROGENOUS CONSTITUENTS OF WATER MELON SEEDS (*CITRULLUS VULGARIS*)

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THIS study was undertaken for the following reasons: (i) The seeds of the water melon are of some nutritional importance as they form an article of diet in certain parts of India. (ii) They are a potent source of urease [Damodaran & Sivaramakrishnan, 1937] and a study of the individual proteins present is of obvious interest in view of the suggested protein nature of this enzyme [Sumner, 1932]. From the point of view of their use in urea determinations it is also necessary to ascertain the presence among the non-protein nitrogenous constituents of the urea-producing bases arginine and canavanine. (iii) Citrulline was isolated by Wada [1930] from water melon fruits and the possibility existed that this interesting amino-acid might be present, free or combined, in the seeds.

The investigation followed conventional lines except that some experiments were carried out to ascertain the nature of the non-protein and non-extractable nitrogenous material.

The chief protein of the seed was readily isolated in crystalline condition from the NaCl extract in yields representing about 27 % of the weight of the fat-free seed meal. A glutelin was prepared in the usual way from the alkaline extract and amounted to 0.9 % of the meal. In the preparation of the glutelin, innovations suggested recently for cereals [Jones & Csonka, 1927; 1928], based upon the separation of globulin and glutelin from an alkaline extract containing both by fractional precipitation with ammonium sulphate, were tried without success, only mixtures of the two proteins being obtained by this method. It was found that the water melon globulin had an isoelectric point of pH 6.5 and that it was precipitated from a solution in 0.2 % NaOH by ammonium sulphate in a concentration as low as 0.0125 of saturation; thus the two criteria suggested by Jones & Csonka for the differentiation of globulin from glutelin do not appear to be of general validity.

Of the nitrogen left in the seed meal after removal of material soluble in water and NaCl only a fraction (16.5 %) was extractable with 0.2 % NaOH. On digesting the residue after NaCl extraction with pepsin it was found that nearly 90 % of the nitrogen went into solution, indicating that the unextracted material was in any case protein in nature. Further information about this fraction was obtained by submitting to acid hydrolysis the residue after NaCl extraction as well as the residue after treatment with NaOH. A large amount of humin material was naturally formed. But on analysing the two filtrates by the nitrogen distribution method it was found that the results were sufficiently in agreement with each other and with those for the isolated glutelin to justify the conclusion that the nitrogenous matter unextractable by NaOH was of the same nature as the isolated glutelin.

Neither the globulin nor the glutelin possessed any urease activity. But the aqueous extracts of the seeds contained small quantities of a protein which could be precipitated at about pH 4.5 and which in the crude state showed strong urease activity. On account of the difficulty experienced in obtaining purified preparations with constant activity further study of this protein had to be reserved for a separate investigation. The non-protein-N of the aqueous extract was found to consist mainly of complex material (proteoses and peptones), simple substances not precipitable by phosphotungstic acid accounting only for a small fraction of the total N extractable by water. The amount of free arginine present was extremely small (5 mg. in 100 g. of fat-free meal) and therefore incapable of causing significant error in urea determinations in which water melon seeds are used as the source of urease.

EXPERIMENTAL

1. Preparation of material

Analysed according to the standard methods of the Association of Official and Agricultural Chemists, the seed contained on the average 5.5 % moisture, 3.8 % ash, 5.5 % of ether-extractable fat and 5.4 % of N. For all extractions the defatted meal was used. The shell-free seeds were ground up in an end-runner to a fine pasty mass, shaken up five or six times with acetone, washed with light petroleum at the pump and dried in a current of air at room temperature. This material contained 11.8 % moisture and 11.73 % N (on dry weight).

2. Nitrogen partition in different protein solvents

The defatted material was exhaustively extracted with water, 10 % NaCl and 0.2 % NaOH in succession. Precipitation with trichloroacetic acid was used for distinguishing between protein and non-protein-N in the extracts (Table I).

Table I. N partition in different solvents

	Total N in extract (as % of N of seed meal)	Non-protein-N (as % of total N of extract)
Water	12.64	50.15
10 % NaCl	77.91	5.98
0.2 % NaOH	1.56	—
Residue	7.83	—

3. Aqueous extract

(a) *Fractionation according to Wasteneys and Borsook.* A water extract of the meal (containing about 0.7 g. N per 100 ml.) was analysed according to the scheme of fractionation of Wasteneys & Borsook [1924] for incomplete protein hydrolysates (Table II). Instead of the "sub-peptone" fraction of these authors, which was obtained by precipitation of the filtrate from tannic acid treatment with alcohol, material precipitable by phosphotungstic acid in the presence of 5 % H₂SO₄ was determined.

The residual filtrate, containing presumably amino-acids (other than basic) and simple peptides not precipitable by phosphotungstic acid, gave positive reactions with ninhydrin, Folin's phenol reagent (tyrosine and tryptophan) and Folin's uric acid reagent (cystine). Tests for citrulline (dimethylamino-benzaldehyde and vanillin hydrochloric acid [Wada, 1930]), canavanine (irradiated nitroprusside [Kitagawa & Yamada, 1932]), and tyrosine (Millon's reaction) were negative.

Table II. *Nature of the nitrogenous constituents in the aqueous extract*

Form	Reagent	Nitrogen (as % of total N in extract)
Protein	Trichloroacetic acid	49.86
Metaprotein	Adjustment to pH 6	Nil
Proteose	Sodium sulphate	28.05
Peptone	Tannic acid	8.93
Basic	Phosphotungstic acid	0.63
Residual	—	12.52

In the filtrate from the tannic acid precipitate arginine was determined by the method of Klein & Taubock [1932]. Excess tannin was removed by means of lead and the latter by means of H_2S . The quantity of arginine found corresponded to 4.88 mg. per 100 g. of seed meal. The solution gave no tests for citrulline or canavanine.

(b) *N distribution after hydrolysis.* The aqueous extract was concentrated *in vacuo* to contain about 600 mg. N per 100 ml. It was refluxed for 16 hr. with 2 vol. of conc. HCl and the main forms of N determined (Table III).

Table III. *Nitrogen distribution on the hydrolysed aqueous extract*

	% of total N
Amide-N	8.46
Humin-N	0.52
Basic amino-N	15.66
Basic non-amino-N	20.81
Monoamino-N	47.95
Non-amino-N	5.45

4. Sodium chloride extract

(a) *Preparation of the globulin.* The defatted meal, after thorough water extraction, was extracted twice with warm 10 % NaCl (5 ml. for each g. of seed meal) by shaking for 2 hr. each time and the combined extracts filtered through paper pulp. The clear filtrate which had a pH of 6.5 was diluted with vigorous stirring with 15 times the volume of distilled water at 40° when immediate precipitation of the protein took place. After allowing to settle overnight in the ice chest the precipitate was separated by centrifuging, redissolved in the minimum amount of 10 % NaCl at 40°, filtered through pulp and reprecipitated by dilution as before. The protein was separated on the centrifuge, washed free from chloride using ice-cold distilled water saturated with CO_2 for the first two washings and graded strengths of acetone afterwards, finally dehydrated with pure acetone and dried at room temperature. The twice precipitated sample which represented 27 % by weight of the defatted material contained 18.6 % N on moisture-free basis and was already pure. Further treatment by dissolving in NaCl and reprecipitation by dilution effected no change in the nitrogen content. The pure protein had an ash content of 0.07 % and analysed as follows: C, 48.12; H, 6.06; N, 18.60; S, 1.02; O, 26.20 %. Molisch's test and the test for phosphorus were negative.

(b) *Crystallization of the globulin.* The protein separating out from the NaCl solution on dilution was in the form of spheroids. Dialysis also yielded a similar product. Crystalline preparations were however readily obtained by the following method: 10 g. of the defatted material were added with vigorous stirring to 160 ml. of 2 % NaCl at 60° and immediately filtered by suction. The protein which began to separate in the filter flask was redissolved by warming to 50° in a water bath, the solution again filtered rapidly, placed in a water bath

at 50° and allowed to cool down slowly to room temperature and then left overnight in the ice chest. The globulin which separated out was composed wholly of microscopic octahedral crystals.

(c) *Nitrogen distribution.* The Van Slyke procedure was followed with the following modifications:

(i) Amide-N was determined by distillation for 4½ min. in the Parnas-Wagner apparatus.

(ii) The dicarboxylic acid-N was estimated according to Damodaran [1931].

(iii) The precipitate of the bases with phosphotungstic acid was dissolved in Winterstein's acetone-water mixture (4 : 3 vol.) and the phosphotungstic acid removed by means of baryta. No solubility correction was applied to the values for the constituents of this fraction.

(iv) Arginine was determined in the solution of the bases, after removal of acetone by distillation and of Ba by H₂SO₄, by the colorimetric method of Klein & Taubock [1932]. Arginine was also determined in a separate hydrolysate without treatment with phosphotungstic acid by three independent methods, viz. by colorimetry [Klein & Taubock, 1932], by precipitation with flavianic acid [Kossel & Gross, 1924] and by means of arginase [Hunter & Dauphinee, 1930]. All three methods yielded concordant results and the average was in excess of the value obtained from the phosphotungstic acid precipitate by an amount that could be accounted for by the solubility factor for arginine phosphotungstate as given by Van Slyke.

(v) Histidine was estimated in the basic fraction both by the usual calculation from the non-amino-N and by a colorimetric method [Hanke & Koessler, 1920]. The value obtained by the two methods agreed within the limits of experimental error.

(vi) No cystine or sulphur was found in the solution of the bases [cf. Damodaran & Sivaswamy, 1936]. Cystine was estimated in a separate hydrolysate by the method of Folin & Marenzi [1929], as modified by Tompsett [1931], and also by the Sullivan method, as modified by Prunty [1933], pure cystine being used as standard in both cases.

In addition to the amino-acids included in the usual Van Slyke scheme tyrosine and tryptophan in the protein were determined according to Folin & Marenzi [1929]. The results are given in Table IV.

Table IV. *Analysis of the globulin*

	% of total N	
	N distribution	Separate determinations
Amide	8.09	—
Humin	0.42	—
Dicarboxylic acid	17.11	—
Arginine	28.03	30.52*; 30.05†; 29.95‡
Histidine	1.47; 1.42§	—
Cystine	(Not found in the P.T.A. precipitate)	0.78‡; 0.76¶
Lysine	5.23	—
Monoamino-monocarboxylic acid	35.71	—
Non-amino	4.81	—
Tyrosine	—	1.92
Tryptophan	—	1.37

* Klein & Taubock [1932].

† Hunter & Dauphinee [1930].

‡ Tompsett [1931].

§ Kossel & Gross [1924].

¶ Hanke & Koessler [1920].

|| Prunty [1933].

5. *Sodium hydroxide extract*

(a) *Preparation of the glutelin.* 200 g. of the defatted seed meal were extracted with water and NaCl solution successively until no more protein was removed, as shown by the absence of turbidity in the final extracts on the addition of trichloroacetic acid. The residue (39 g. containing 5.66% N by dry weight), after being washed free from NaCl with water, was extracted eight times by shaking with 0.2% NaOH, 50 ml. lots being used for each extraction which lasted an hour. At the end of each extraction the liquid was centrifuged and the centrifugate filtered through paper pulp. To the clear filtrate 0.2% HCl was slowly added with stirring till the pH of the solution was 5.9 (chlorophenol red), which was found by trial on a number of samples to be the point of maximum precipitation.

The absence of any precipitate or turbidity on adjusting the pH to 6.5 (the isoelectric point of the globulin) can be taken to prove that no globulin was present in the solution. Each extract was brought to pH 5.9 with acid immediately after filtration in order to prevent the protein being left in long contact with alkali. The precipitates were combined, redissolved in the minimum amount of dilute alkali and reprecipitated after filtration as before; this process was repeated three times more, a sample being washed and dried for analysis after each precipitation. The five times-precipitated sample was used for elementary analysis and the three times-precipitated one for nitrogen distribution. The nitrogen content (% of dry weight) after successive precipitations was 14.95, 15.02, 15.11, 15.15 and 15.15 respectively, the values being unaffected after the third precipitation.

The protein did not exhibit any tendency to disperse in the water used for washing it free from chloride. It was dehydrated with acetone and dried at room temperature under a fan. The yield of the glutelin was 1.76 g. It gave a negative Molisch test but contained phosphorus. The five times-precipitated sample after air-drying had a moisture content of 11.51% and 0.59% ash (calculated on the dry weight of protein). After drying *in vacuo* over P_2O_5 at 100° it analysed as follows: C, 47.80; H, 5.98; N, 15.15; S, 0.98; P, 0.12; O, 29.97%. Sulphur was determined by two methods, by fusion with sodium peroxide and by Pirie's method [1932], the values obtained being 0.95 and 0.98%. Phosphorus was determined by the colorimetric method of Fiske & Subbarow [1925] on a sample of the protein which was extracted with methyl alcohol, acetone, light petroleum and finally ether.

Table V. *Analysis of the glutelin*

	% of total N	
	N distribution	Separate determinations
Amide	7.26	—
Humin	2.06	—
Dicarboxylic acid	13.15	—
Arginine	14.85*	15.18*
Histidine	4.41	—
Cystine	0.17†	2.85‡; 2.75†
Lysine	4.73	—
Monoamino monocarboxylic acid	45.43	—
Non-amino	7.89	—
Tyrosine	—	1.63
Tryptophan	—	1.08

* Klein & Taubock [1932].

† Prunty [1933].

‡ Tompaett [1931].

(b) *Nitrogen distribution.* The procedure adopted was the same as in the case of the globulin with slight differences. 1 g. samples of the protein were taken for hydrolysis instead of 3 g., and all volumes and reagents correspondingly reduced. The precipitate of the bases with phosphotungstic acid was dissolved in NaOH and the phosphotungstic acid removed by means of baryta. Arginine determinations carried out in this solution and in a separate hydrolysate by the method of Klein & Taubock gave values which did not differ appreciably from each other.

6. Unextracted nitrogenous material

(a) *Action of pepsin.* As only one-sixth of the nitrogen of the residue after NaCl extraction was extractable by dilute alkali the action of pepsin on this residue was tried to ascertain the nature of the nitrogenous material present. 5 g. of the residue after sodium chloride extraction were ground intimately with 0.05 N HCl, and made up to 250 ml. with 0.05 N HCl containing 0.25 g. of pepsin. After standing at 37° for one week the digest was filtered, the insoluble material washed free from chloride, and the filtrate and washings analysed for nitrogen content. 89.31 % of the nitrogen in the residue was found to have gone into solution under the action of pepsin. The insoluble material weighed 1.4 g., representing 5.98 % of the weight of seed meal, and contained 1.83 % N (on dry weight).

(b) *Hydrolysis of the residues.* As the digestion with pepsin also failed to bring the whole of the nitrogen into solution, it was thought desirable to analyse the residues after NaCl extraction and after extraction with NaOH, and compare the values with those obtained with the glutelin. The results are given in Table VI.

Table VI. *Analysis of the residues before and after extraction of the glutelin*

	% of total N	
	Residue after NaCl extraction	Residue after NaOH extraction
Amide	7.30	7.43
Humin	3.41	3.43
Arginine	14.98*	14.75*
Histidine	4.11	4.49
Cystine	(Not found in the P.T.A. precipitate)	
Lysine	4.94	4.67
Monoamino	57.40	57.96
Non-amino	7.45	7.06
Tyrosine	1.67	1.74
Tryptophan	1.00	1.004
Cystine	2.98†; 2.71‡	2.92†; 2.70‡

* Klein & Taubock [1932].

† Tompsett [1931].

‡ Prunty [1933].

SUMMARY

1. The nitrogen of water melon seeds is shown to be made up of: glutelin, 9.4 % (extractable 1.6 %, not extractable 7.8%); globulin, 73.2 %; water-soluble protein, 6.3 %; proteoses, 3.5 %; peptones, 1.1 %; material precipitable by phosphotungstic acid, 0.1 %; simpler substances not precipitable by phosphotungstic acid, 1.6 %.

2. The globulin and the glutelin have been isolated in pure, the former in crystalline condition, and the most important amino-acids in the two proteins determined. Independent determinations of arginine and histidine have borne

out the accuracy of the values for these amino-acids obtained by the nitrogen distribution method.

3. The seeds do not contain canavanine or citrulline, either free or combined, and only traces of free arginine.

Our thanks are due to Prof. M. Damodaran for his interest in the work.

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CLVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LXII. THE CRYSTALLINE COLOURING MATTERS OF SPECIES IN THE *ASPERGILLUS GLAUCUS* SERIES. PART II

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GOULD & RAISTRICK [1934] reported the isolation of three new crystalline colouring matters which they obtained by extraction with light petroleum of the dried mycelium of 25 species in the *Aspergillus glaucus* series. The colouring matters and the empirical formulae provisionally assigned to them were: (a) flavoglaucin, $C_{19}H_{28}O_3$, lemon-yellow needles; (b) auroglaucin, $C_{19}H_{22}O_3$, orange-red needles; (c) rubroglaucin, $C_{16}H_{12}O_5$, ruby-red needles. Further investigation of these colouring matters has confirmed the views expressed by Gould & Raistrick in the cases of flavoglaucin and auroglaucin and provisional structural formulae have now been assigned to them [Raistrick *et al.* 1937; Cruickshank *et al.* 1938].

The case is different with rubroglaucin. In the earlier work only very small amounts of crude rubroglaucin were available for purification and chemical examination, but investigation of this material by Raistrick *et al.* [1937] appeared to confirm the results of Gould & Raistrick [1934] and to indicate that rubroglaucin is a dihydroxymonomethoxymethylanthraquinone. In order to settle this point larger quantities of rubroglaucin have now been isolated from more recently prepared mycelium of the same strain of *Aspergillus ruber* (Spieckermann & Bremer) Thom & Church as was used by Gould & Raistrick three years previously for the preparation of this colouring matter. Exhaustive fractionation of the crude red pigment obtained resulted in the isolation of two hydroxyanthraquinones, i.e. physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, and a hitherto undescribed trihydroxymonomethoxymethylanthraquinone, of at present undetermined molecular constitution, for which the name *erythroglaucin* is proposed. The question then immediately arose as to whether this finding was to be explained (a) by a change in the metabolic processes of this strain of *A. ruber* after 3-4 years' cultivation on laboratory media, or (b) by the fact that the substance previously described as rubroglaucin is in fact not an entity but a mixture of physcion and erythroglaucin which is unusually difficult to separate into its components. Possibility (a) seems to be ruled out by the facts that both physcion and erythroglaucin have now been isolated from the mycelium of the Gould & Raistrick strain of *A. ruber* when this was grown on two different media and after incubation periods of 28, 41 and 100 days, and also from a number of other different strains of *A. ruber* obtained from widely different sources. No *pure* substance having the properties

recorded for rubroglaucin was encountered though mixtures of physcion and erythroglaucin having these properties were observed. Possibility (b) was shown to be correct by the actual isolation of physcion and erythroglaucin from a very small sample of rubroglaucin originally prepared by Dr B. S. Gould in 1934. Hence rubroglaucin must now be regarded as a mixture of physcion and erythroglaucin and the word rubroglaucin becomes a *nomen nudum*.

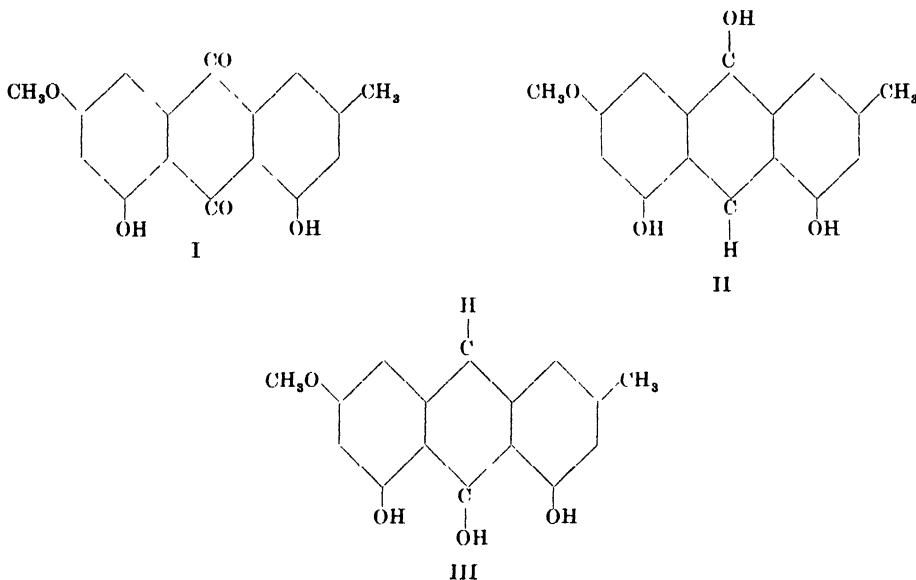
Gould & Raistrick [1934] suggested that the 25 species of moulds in the *A. glaucus* series which they examined might be conveniently divided into three groups on the basis of the pigments, soluble in light petroleum (B.P. 40–50°), which they contain. Group I, containing 13 species, consisted of those species giving flavoglaucin and auroglaucin only; group II contained 2 species which give flavoglaucin, auroglaucin and an unidentified red pigment; group III contained 10 species which give flavoglaucin and at least one red pigment which in four cases was believed to be rubroglaucin. No auroglaucin was isolated from members of group III. It was also reported that, in addition to the highly coloured pigments flavoglaucin, auroglaucin and rubroglaucin, there were present in the light petroleum extracts of species in groups II and III other much less coloured, if not indeed colourless, extractives which were not examined further. Hence, in view of our more recent findings on rubroglaucin, it obviously became of interest to re-examine the species in groups II and III in more detail than was previously possible. This has now been done and the results obtained form the major portion of the present communication.

The following species, representing all those in groups II and III, except *A. lovaniensis* Biourge which has become atypical, together with certain additional strains of *A. ruber*, were grown on the same synthetic medium and under the same cultural conditions as were employed previously: Group II, *A. oriolus* Biourge, *A. albidus* Spegazzini; Group III, six different strains of *A. ruber* (Spieckermann & Bremer) Thom & Church, *A. ferrugineus* Fuckel, *A. glaucus* mut. *alba* Blochwitz, *A. umbrosus* Bainier & Sartory, *A. mongolicus* Biourge, *A. echinulatus* (Delacroix) Thom & Church, *A. herbariorum* series *minor* (Mangin) Thom & Church, *A. mutabilis* Bainier & Sartory and *A. repandus* Bainier & Sartory. The dried mycelium was exhaustively extracted with light petroleum and the extract, after removal of fat, was repeatedly and very carefully fractionated from different solvents.

It may be said at once that the apparent differences between groups II and III previously suggested now largely disappear. All the species, with the possible exception of *A. echinulatus* where the evidence is doubtful, gave flavoglaucin in larger or smaller amounts. Hence the view is confirmed that this pigment is specific for and constantly present in all members of the *A. glaucus* series. On the other hand auroglaucin has now been detected in three species in group III, i.e. *A. ruber* (in two strains out of six), *A. glaucus* mut. *alba* and *A. ferrugineus* and confirmed in both members of group II, i.e. *A. oriolus* and *A. albidus*. Hence, in view of the difficulty of detecting small amounts of auroglaucin in the presence of relatively large amounts of the other colouring matters, we now regard the isolation of auroglaucin as being not sufficiently certain to justify, in itself, the separation of groups II and III. The essential homogeneity of groups II and III is, moreover, seen in the results of the examination of their hydroxyanthraquinone content. In no species examined was any evidence forthcoming of the presence of a *pure* substance having the properties of rubroglaucin though in many cases fractions, which subsequently proved to be mixtures, were isolated approximating in colour reactions, M.P. etc. to those previously recorded for rubroglaucin. In all the species examined, physcion

(4:5-dihydroxy-7-methoxy-2-methylanthraquinone) was detected and was identified in some cases by direct comparison with a sample prepared from the lichen *Xanthoria parietina* (L.) and in others by direct comparison of their diacetates. With the single exception of *A. mutabilis*, erythroglaucon was also isolated from all the species examined.

In addition to physcion and erythroglaucon we have also isolated two other substances from five of the mould species examined, i.e. *A. oriolus* in group II, *A. mongolicus*, *A. mutabilis*, *A. herbariorum* and two strains of *A. ruber* in group III. These substances, which are of some biological interest, are both reduction products of physcion and will be referred to as physcion anthranol A, m.p. 260°, and physcion anthranol B, m.p. 181–2°. We have also prepared physcion anthranol B by reduction of physcion and have converted both physcion anthranol A and physcion anthranol B into physcion by oxidation. The probable relation of the two anthranols to physcion (structure I) is given in structures II and III though it is not possible to say which of these structures is to be referred to anthranol A and which to anthranol B.



The occurrence of physcion and the two anthranols in the mycelium of the above-mentioned moulds adds one more example to the growing list of similar substances, reported from these laboratories as metabolic products of the lower fungi, which suggest that these substances probably play a part as oxidation-reduction systems in the vital processes of the moulds which produce them.

Physcion is widely distributed in nature in plants of very different types. It has been known for many years, formerly under the name parietin, as one of the so-called lichen acids, occurring especially in several species of *Xanthoria* and *Placodium*. It was isolated in small amounts by Raistrick *et al.* [1937] from crude flavoglaucon prepared from *A. glaucus* Link, a species which was included by Gould & Raistrick [1934] in group I. As long ago as 1894 it was observed by Perkin & Hummel, and described by them as emodin monomethyl ether, in the root-bark of *Ventilago madraspatana* Gärtn., a large climbing shrub common in Southern India and Ceylon. Of even greater interest, however, from the point

of view of the present communication, is the fact that Perkin & Hummel isolated, in addition to physcion, two anthranols A and B, which melted respectively at 260 and 173°, both of which they converted by oxidation into physcion. Although we have not been able to compare directly our two anthranols, melting respectively at 260 and 181–2°, there seems to be little doubt that the mould anthranols are identical with those from *Ventilago madraspatana*. So far as we are aware physcion anthranol A, m.p. 260°, has not been reported from any other sources, but physcion anthranol B, m.p. 181–2°, has been described by several other workers, notably by Hesse [1912; 1917] who isolated it (m.p. 181°, 184°) from chrysarobin ("Goa powder" of commerce), a crystalline exudation from the wood of various species of *Andira* especially *Andira araroba* Ag. The same worker [1895] also obtained it (m.p. 180–2°) by the reduction of physcion.

Erythroglauzin, $C_{16}H_{12}O_6$, deep red plates, m.p. 205–6°, is a monomethyl ether of a tetrahydroxymethylanthraquinone. It contains a methyl group and gives a triacetate but is not the methyl ether of cynodontin, itself a tetrahydroxymethylanthraquinone, and present in the mycelium of the mould *Helminthosporium cynodontis* Marignoni [Raistrick *et al.* 1933], since its trimethyl ether is not identical with the tetramethyl ether of cynodontin.

EXPERIMENTAL

History of cultures used

The following species were used in the experimental work described below:

Catalogue no.	Species	Source
(1) No. 6	<i>A. ruber</i> (Spieckermann & Bremer) Thom & Church	Isolated by Mr G. Smith in 1926 from mildewed cotton cloth
(2) No. 6 (Gould)	" "	Previous strain, maintained in culture in America by B. S. Gould from 1934 onwards and returned here, 1938
(3) No. 23	" "	Isolated by Mr G. Smith in 1926 from cotton yarn
(4) A. 20	" "	Received from Centraalbureau voor Schimmelcultures, Baarn, 1937. Miss Church's strain
(5) A. 22	" "	Received from Centraalbureau voor Schimmelcultures, Baarn, 1937. Pollacci (1931) strain
(6) A. 47	" "	Received from Mr L. D. Galloway, 1937. Isolated in India
(7) H.A. 5	" "	Isolated by Mr G. Smith in 1938 from infected hops
(8) Ac. 39	<i>A. ferrugineus</i> Fuckel	Received from Baarn, 1923
(9) A. 16	<i>A. glaucus</i> mut. <i>alba</i> Blochwitz	Received from Baarn, 1933
(10) A. 18	<i>A. umbrosus</i> Bainier & Sartory	Received from Baarn, 1933
(11) A. 36	<i>A. mongolicus</i> Biourge	Received from Prof. Ph. Biourge, Louvain, 1933
(12) A. 28	<i>A. echinulatus</i> (Delacroix) Thom and Church	Received from Prof. Ph. Biourge, Louvain, 1933
(13) No. 96	<i>A. herbariorum</i> series <i>minor</i> (Man- gin) Thom & Church	Received by Mr G. Smith in 1927 from Shirley Institute
(14) A. 40	<i>A. mutabilis</i> Bainier & Sartory	Received from Dr Charles Thom in 1933
(15) A. 17	<i>A. repandus</i> Bainier & Sartory	Received from Baarn, 1933
(16) A. 30	<i>A. oriolus</i> Biourge	Received from Prof. Ph. Biourge, 1933
(17) A. 35	<i>A. albidus</i> Spegazzini	Received from Prof. Ph. Biourge, 1933

With the exception of cultures 3, 4, 5, 6 and 7 all these cultures had been examined previously by Gould & Raistrick [1934].

Cultural conditions

Two synthetic media, previously used by Gould & Raistrick, were employed throughout this work.

Medium A. Glucose, 1750 g.; diammonium tartrate, 75.6 g.; KH_2PO_4 , 35.0 g.; KCl, 17.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 17.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.35 g.; distilled water, 35 l.

Medium B. As for medium A except that 378 g. of diammonium tartrate was used in place of 75.6 g.

In order to avoid decomposition the ammonium tartrate was sterilized separately, the details being given in Gould & Raistrick's paper [1934, p. 1643]. With all species examined 100 one-litre conical flasks, each containing 350 ml. of sterile medium, were inoculated with a spore suspension of the chosen organism washed off beer-wort agar slopes, 4 flasks being sown with one slope. The flasks were incubated at 24° for periods of 28 to 100 days, until metabolism of the glucose present had become very slow. At the end of this time the medium was covered with a felt of mycelium varying in colour from greyish-brown with some species, through rust colour to a bright reddish-brown in others. The mycelium was then separated by filtration, washed carefully with distilled water, pressed, dried in a vacuum oven at $35\text{--}40^\circ$ and ground in a coffee mill.

Extraction of the mycelium and fractionation of the colouring matters

Six different substances—flavoglauca, auroglauca, erythroglauca, physcion and the physcion anthranols A and B—may be present in the mycelium under investigation, and since the total number of colouring matters present in any one species, and also their relative proportions, vary from species to species, it is obviously not possible to give the exact details of the methods of fractionation followed in every case. The general procedure, modified in some details to suit individual cases, was as follows. The ground mycelium was in all cases extracted exhaustively with light petroleum (B.P. $40\text{--}50^\circ$) in a Soxhlet apparatus until the extract became colourless. The time required for complete extraction varied from 8 to 22 hr. The light petroleum extracts were held at 0° until separation was complete and the solid separating was filtered, washed with cold light petroleum and dried. The filtrate and washings were combined, evaporated and further crops of solid material were united with the first crop. The final mother liquor, on evaporation, gave varying amounts of fat. The total crude pigment obtained varied from a minimum of 0.48 g. with *A. ruber* Cat. No. 23 to a maximum of 31.0 g. with *A. mongolicus*.

The combined crude colouring matters were now ground with cold methanol in which flavoglauca is readily soluble and auroglauca moderately soluble, while erythroglauca, physcion and the physcion anthranols are only very slightly soluble; 8–10 ml. of methanol per g. of crude colouring matter were used. The undissolved material, usually bright red in colour, was separated by filtration (for further treatment see later) and the filtrate and washings, containing flavoglauca and auroglauca, evaporated to dryness in air. The dry residue was now extracted with boiling light petroleum (B.P. $50\text{--}60^\circ$) in which flavoglauca is much more readily soluble than auroglauca. The undissolved residue consisting of crude auroglauca was purified by crystallization from ethyl acetate. Flavoglauca was recovered from the light petroleum solution and was purified by crystallization from light petroleum (B.P. $40\text{--}50^\circ$) and 75% alcohol.

The crude red pigment obtained on Medium A varied in weight from 0.06 g. with *A. ferrugineus* to 10.42 g. with *A. oriolus* per 100 flasks, and in m.p. from 142 to 155° in the case of *A. repandus* to 182–7° in the case of *A. ferrugineus*. It may contain two or more of the substances erythroglaucon, physcion and the physcion anthranols A and B and was subjected to prolonged fractional crystallization. Ethyl acetate was used almost exclusively for the separation and purification of erythroglaucon and physcion, benzene and toluene being used for the purification of the anthranols. Erythroglaucon and physcion were separated by making use of the facts that physcion is much more soluble in warm ethyl acetate than is erythroglaucon, and that while erythroglaucon usually crystallizes in characteristic flat plates, physcion generally separates in slender needles. Actual details of each crystallization vary from fraction to fraction, but the general principle of the method used is as follows: the solution of the red pigments in hot ethyl acetate was allowed to cool very slowly and the type of crystal separating was followed by examination with a powerful hand lens. The first crystals to appear were usually flat plates (erythroglaucon) and these were separated by filtration of the hot solution as quickly as possible so as to minimize admixture with needle-shaped crystals (physcion). The mother liquors, on cooling to room temperature, deposited a mixture of erythroglaucon and physcion and the filtrate from these on concentration gave mainly physcion. At every stage the m.p., colour reaction with cold conc. H_2SO_4 , and crystalline form were determined. Consideration of each of these tests decided which fractions should be combined and the fractional crystallization was then repeated. The process is a long and tedious one but leads eventually to pure specimens of erythroglaucon and physcion. A mixture of about 30 % erythroglaucon and 70 % physcion is very difficult to separate into its constituents, melts at about 180°, looks homogeneous and gives an intense Tyrian purple colour with conc. H_2SO_4 . Such a mixture corresponds closely to the rubroglaucon of Gould & Raistrick [1934].

The fractionation of the crude red colouring matters is rendered very much more difficult if physcion anthranols A and B are present. Physcion anthranol A forms pale yellow needles, m.p. 260°, is much less soluble in organic solvents than any of the other colouring matters and hence can be fairly readily isolated. Physcion anthranol B, m.p. 181–2°, on the other hand, has physical properties which are almost identical with those of physcion. Hence it is only isolated from a mixture with physcion by a long and tedious fractional crystallization from ethyl acetate and benzene. So difficult is this separation that our specimens of physcion anthranol B have still a pale pink colour, whereas specimens of physcion anthranol B, prepared by the reduction of physcion with zinc dust in glacial acetic acid are yellow in colour, but a mixture of the two preparations shows no depression in m.p. The pinkish colour of specimens of mould physcion anthranol B also persists after fractional sublimation in a high vacuum at 130°.

Composition of the mixed colouring matters isolated from the different species

The experimental results obtained with all the species examined are given in Table I in which all weights are expressed as g. per 100 flasks of culture medium. The temperature of incubation was 24° throughout. It should be noted that the pigments described are only those which can be extracted with light petroleum, b.p. 40–50°. Many species, after complete extraction with light petroleum, are still highly coloured and the composition of these light petroleum-insoluble colouring matters will form the subject of a future communication. All species

Table I

Exp. no.	Species used and catalogue no.	Medium	Incu- bation period days	% re- sidual glucose	My- celium g.	Total pig- ment g.	Crude flavo- glau- cous g.	Auro- glau- cous g.	Total crude red pig- ment g.	M.P. crude red pig- ment °C.	Pure ery- thro- glau- cous g.	Mainly ery- thro- glau- cous g.	Pure phy- tho- scion g.	Mainly phy- tho- scion g.
1	<i>A. ruber</i> (No. 6)	A	28	2.60	113	7.73	6.58	0.10	1.05	167-72	0.14	0.04	0.29	0.15
2	<i>A. ruber</i> (No. 6)	A	40	1.62	113	4.32	3.77	—	0.55	173-8	0.06	—	0.18	0.04
3	<i>A. ruber</i> (No. 6) (Gould)	A	41	1.86	124	5.85	4.80	—	1.05	166-71	0.12	—	0.33	0.16
4	<i>A. ruber</i> (No. 6)	B	41	0.20	157	7.71	7.05	—	0.66	170-85	0.08	0.15	0.10	0.02
5	<i>A. ruber</i> (No. 6)	A	100	1.84	123	7.35	6.37	0.020	0.96	177-81	0.065	0.195	0.41	—
6	<i>A. ruber</i> (No. 23)	A	42	2.57	100	0.48	0.27	—	0.21	176-80	—	0.08	Trace	—
7	<i>A. ruber</i> (A 20)	A	37	3.10	42	3.38	3.28	—	0.10	177-81	0.025	0.025	0.02	—
8	<i>A. ruber</i> (A 22)	A	39	3.10	126	3.59	2.36	—	1.23	160-5	0.32	—	0.015	0.04
9	<i>A. ruber</i> (A 47)	A	43	3.00	109	5.43	5.12	—	0.31	165-8	0.07	—	0.022	0.04
10	<i>A. ruber</i> (H A. 5)	A	37	1.76	133	10.35	8.92	0.20	1.23	170-5	0.10	0.04	0.44	—
11	<i>A. ferrugineus</i> (Ac 39)	A	48	2.15	90	3.84	3.76	0.02	0.06	182-7	0.04	—	—	0.01
12	<i>A. glaucus</i> mut. <i>alba</i> (A 16)	A	51	3.10	117	11.6	9.64	0.70	1.26	160-4	0.012	—	0.10	0.09
13	<i>A. umbrosus</i> (A 18)	A	45	2.20	108	3.35	3.22	—	0.13	180-6	0.006	—	0.007	—
14	<i>A. mongolicus</i> (A 36)	A	75	0.72	209	19.0	16.70	—	2.30	142-8	0.30	—	0.414	—
15	<i>A. mongolicus</i> (A 36)	B	96	0.076	298	31.0	27.16	—	3.84	171-7	0.075	0.075	1.96	0.61
16	<i>A. echinulatus</i> (A 28)	A	44	2.95	141	0.91	—	—	0.84	163-8	0.21	—	0.11	—
17	<i>A. herbariorum</i> (No. 96)	A	47	1.75	130	1.75	0.85	—	0.90	160-4	0.03	—	0.02	0.05
18	<i>A. mutabilis</i> (A 40)	A	64	0.75	148	6.26	0.76	—	5.50	160-5	—	—	0.44	0.33
19	<i>A. mutabilis</i> (A 40)	B	84	0.075	120	1.31	0.53	—	0.01	202-5	—	—	0.01	—
20	<i>A. repandus</i> (A. 17)	A	35	1.70	119	11.34	11.08	—	0.26	142-55	0.11	—	0.02	0.02
21	<i>A. oroidus</i> (A 30)	A	53	1.74	210	28.28	17.85	Trace	10.42	160-6	0.21	0.05	—	0.10
22	<i>A. albidus</i> (A 35)	A	70	2.43	157	20.77	19.45	1.20	0.12	180-1	0.022	0.01	0.02	0.01

were grown on culture medium A, which was the medium used by Gould & Raistrick [1934] in their original investigation of the red colouring matters. In certain additional instances (see col. 3) culture medium B was also used (for composition of media A and B see p. 1295).

The following conclusions may be drawn from the results given in Table I.

(1) *Flavoglaucin* (see col. 8). Flavoglaucin was isolated from all the species examined with the exception of *A. echinulatus*, thus confirming the view expressed by Gould & Raistrick [1934] that this colouring matter may be regarded as specific for species in the *A. glaucus* series. In almost all cases flavoglaucin constituted 80% or more of the total colouring matters extractable with light petroleum. Considerable amounts of flavoglaucin were isolated from almost all species examined and in some instances, e.g. *A. mongolicus*, *A. glaucus* mut. *alba*, *A. repandus*, *A. oriolus* and *A. albidus*, amounted to about 10% or more of the weight of the dry mycelium. The m.p. of the crude flavoglaucin isolated was 80–90° but was raised on purification to that previously recorded for pure flavoglaucin, i.e. 104–5°. Flavoglaucin formation appears to be enhanced on medium B in comparison with medium A (see exps. 2 and 4 for *A. ruber* Cat. No. 6 grown under otherwise identical cultural conditions and exps. 14 and 15 for *A. mongolicus*). This observation is in agreement with similar findings for *A. novus* made by Gould & Raistrick [1934].

(2) *Auroglaucin* (see col. 9). Auroglaucin was isolated, not only from *A. oriolus* and *A. albidus*, as was previously reported by Gould & Raistrick [1934], but also from *A. ruber* Cat. No. 6, *A. ruber* Cat. No. H.A. 5, *A. glaucus* mut. *alba* and *A. ferrugineus*, though in all cases the amounts isolated were small. Hence the view tentatively expressed by Gould & Raistrick that *A. oriolus* and *A. albidus* could be separated from the species in the *A. glaucus* series giving red pigments, because of their ability to produce auroglaucin, must now be abandoned. The auroglaucin isolated was identified in all cases by its colour, crystalline form, m.p. 153° not depressed in any instance on admixture with an authentic specimen, and in one case, that from *A. ruber* Cat. No. H.A. 5, by analysis. (Found: C, 76.10; 76.12; H, 7.42, 7.34%. Calc. for $C_{19}H_{22}O_3$, C, 76.45; H, 7.44%.)

(3) *Erythroglaucin* (see cols. 12 and 13). Erythroglaucin was isolated from all the species examined with the single exception of *A. mutabilis*. This species was grown on both media A and B, but only small amounts of hydroxyanthraquinone colouring matters were formed on either medium and consisted, so far as could be ascertained, almost exclusively of physcion and its anthranols on medium A and physcion alone on medium B. The amounts of erythroglaucin isolated were, in all cases, very small, reaching a maximum of 0.32 g. per 100 flasks with *A. ruber* Cat. No. A 22. The identity of the different specimens of erythroglaucin isolated was established in all cases by their colour reactions with conc. H_2SO_4 and with 2N NaOH and by their m.p. which varied according to purity from a minimum of 195–8° with the specimen from *A. repandus* to a maximum of 204–6° with that from *A. ruber* Cat. No. A 22. Almost all samples melted above 200° and none of them gave any depression on admixture with a specimen isolated from *A. ruber* Cat. No. 6, m.p. 199–201°. The general properties of erythroglaucin are described later (see p. 1300).

(4) *Physcion* (see cols. 14 and 15). Physcion was isolated from all the species examined, in amounts which while not generally large in themselves were usually greater than the amounts of erythroglaucin obtained from the corresponding species. Considerable quantities of pure physcion were isolated from *A. mongolicus* (1.96 g. from 100 flasks) when this species was grown on medium B. The

influence of the medium on the nature and amount of the colouring matters formed is particularly well shown by this species, since when it was grown on medium A only 0.41 g. of pure physcion was isolated. The different specimens of physcion obtained were identified by their colour reactions with conc. H_2SO_4 and with 2N NaOH and by their m.p. Almost all the samples melted in the range $200^\circ \pm 2^\circ$ and none of them gave any depression in m.p. on admixture with the specimen from *A. ruber* Cat. No. H.A. 5 (m.p. 199–200°). The general properties of mould physcion and the proof of its identity with lichen physcion are described later (see pp. 1300 and 1301).

(5) *Physcion anthranol A*, m.p. 260°. This anthranol, the properties of which are described later (see p. 1301), was isolated from the following species, grown in each case on medium A: (a) *A. ruber*, Cat. No. A 47; 0.005 g., m.p. 254–6°. (b) *A. ruber*, Cat. No. 22; 0.015 g., m.p. 253–7°. (c) *A. mongolicus*, 0.023 g., m.p. 253–5°. (d) *A. mutabilis*, 0.08 g., m.p. 253–7°. (e) *A. herbariorum*, 0.02 g., m.p. 253–7°. (f) *A. oriolus*, 0.05 g., m.p. 258–62°. None of the specimens (a) to (e) gave any depression in m.p. on admixture with specimen (f).

(6) *Physcion anthranol B*, m.p. 181–2°. This anthranol, the properties of which are described later (see p. 1301), was identified in each of the species giving physcion anthranol A, except *A. ruber* Cat. No. A 47, and was usually isolated in much larger amounts than physcion anthranol A. The medium in each case was medium A. (a) *A. ruber*, Cat. No. 6; a trace. (b) *A. ruber*, Cat. No. A 22; 0.065 g., m.p. 180–1°. (c) *A. mongolicus*: a trace. (d) *A. mutabilis*; 0.26 g., m.p. 181–2°. (e) *A. herbariorum*; 0.07 g., m.p. 180–1°. (f) *A. oriolus*; 2.44 g., m.p. 180–2°. None of the above specimens gave any depression in m.p. on admixture with a synthetic specimen of physcion anthranol B, m.p. 182°, made by the reduction of physcion. It is of interest that while both physcion anthranols A and B were detected in both *A. mongolicus* and *A. mutabilis* when grown on medium A, neither anthranol could be detected in either of these moulds when grown on medium B.

The total weight isolated of erythroglaucon, physcion and the two anthranols was in general only a relatively small fraction of the weight of crude red pigment fractionated. This is due in part to unavoidable experimental losses and to the presence of considerable amounts of resinous material which could not be crystallized. In one case, i.e. *A. mutabilis* on medium B, a colourless substance crystallizing from ethanol in small rosettes, m.p. 97–100°, was isolated. This substance, which gave a strongly positive Liebermann sterol reaction, was identified as ergosterol palmitate by direct comparison with an authentic specimen isolated from *P. brevi-compactum* Dierckx [Oxford & Raistrick, 1933].

Fractionation of crude red colouring matters prepared by Dr B. S. Gould in 1934. Certain of the fractions of crude red colouring matters prepared by Dr Gould in 1934 and described by Gould & Raistrick [1934] being available were fractionated by the methods described above and gave the following results:

(1) From *A. ruber* Cat. No. 6, wt. 0.63 g., 0.06 g. of erythroglaucon, 0.18 g. of physcion and 0.18 g. of a crude mixture of the two anthranols were isolated. No evidence was obtained of the presence of rubroglaucon.

(2) From *A. mongolicus*, wt. 0.07 g. 0.005 g. of erythroglaucon and 0.004 g. of physcion were isolated. The residues contained small amounts of anthranols as was indicated by the colour reaction with conc. H_2SO_4 .

(3) A mixture of pigments from *A. ferrugineus* and *A. repandus* recorded by Gould & Raistrick [1934, p. 1647] as melting at 193–5°. Wt. 0.01 g. This material was almost pure erythroglaucon and yielded 0.007 g. of erythroglaucon in a single crystallization.

General properties of the colouring matters

(A) *Erythroglaucin*. Erythroglaucin is dimorphic, and although it usually crystallizes in small, regular, square, deep red plates, it sometimes separates in short sharp-pointed red needles. The two forms have the same m.p. and show no depression on mixing. m.p. 205–6°. (Found: C, 63.85, 63.91; H, 3.98, 4.00; CH₃O, 10.0%. C₁₆H₁₂O₆ requires C, 63.98; H, 4.03; 1 CH₃O, 10.3%.) On oxidation with chromic acid (Kuhn-Roth method) evidence of one side-chain methyl group was obtained. (Found: 102.3, 95.7% of 1 CH₃COOH.) It is only slightly soluble in light petroleum, methanol, ethanol and ether, is moderately soluble in cold benzene, chloroform, acetone and ethyl acetate, but is readily soluble on heating. It gives an orange-red solution in organic solvents, the solution having a slight green fluorescence. It gives a pure deep blue colour in dilute solution in cold conc. H₂SO₄ and a purple solution with 2N NaOH which slowly gives a violet precipitate. An alcoholic solution gives the following reactions: (a) with aqueous NaOH or ammonia an intense red-violet colour which is destroyed on the addition of aqueous NaOCl. (b) With the following aqueous solutions; borax, purple; magnesium sulphate, pink; nickel chloride, cerise; ferric chloride, olive brown; copper sulphate, lilac precipitate; lead acetate, no change.

Triacetylerythroglaucin. Erythroglaucin (0.062 g.) was acetylated by the Peterson-West [1927] method by treatment at 37° for 16 hr. with 3 g. of a mixture of pyridine (5 parts) and acetic anhydride (1 part). Acetic anhydride equivalent to 6.1 ml. of N/10 NaOH was fixed. Theoretical for three acetyltable groups, 6.2 ml. The triacetyl-erythroglaucin separating during the estimation was crystallized from acetic acid in clusters of bright yellow rods (0.060 g.), m.p. 225°. (Found: C, 61.96, 61.82; H, 4.28, 4.09; CH₃O, 7.4, 7.2%. C₂₂H₁₈O₉ requires C, 61.95; H, 4.26; 1 CH₃O, 7.3%.)

Erythroglaucin trimethyl ether. A mixture of erythroglaucin (0.063 g.), K₂CO₃ (1 g.), acetone (20 ml.) and dimethyl sulphate (0.5 ml.) was refluxed for 2½ hr. K₂CO₃ (1 g.), acetone (10 ml.) and dimethyl sulphate (0.5 ml.) were now added and the mixture refluxed for a further 4 hr. The mixture was filtered, the acetone removed from the filtrate and the residue was washed with light petroleum and water. Wt. 0.058 g. It crystallized from a mixture of benzene and light petroleum (b.p. 80–100°) in yellow needles, m.p. 187–8°. (Found: C, 66.55, 66.55; H, 5.26, 5.36; CH₃O, 37.0%. C₁₆H₁₈O₆ requires C, 66.63; H, 5.30; 4 CH₃O, 36.3%.) The substance was quite insoluble in aqueous NaOH and gave a deep blue colour with cold conc. H₂SO₄. Erythroglaucin cannot be a monomethyl ether of cynodontin, a metabolic product of *Helminthosporium cynodontis* Marignoni [Raistrick *et al.* 1933] since erythroglaucin trimethyl ether is not identical with the *tetramethyl ether of cynodontin* which was prepared from cynodontin (0.1 g.) by the same method as is described above. Yield, 0.08 g. It was crystallized from benzene in orange needles, m.p. 233–4°, which are insoluble in aqueous NaOH and give a peacock blue colour with cold conc. H₂SO₄. (Found: C, 66.46, 66.51; H, 5.21, 5.35; CH₃O, 36.0%. C₁₈H₁₈O₆ requires C, 66.63; H, 5.30; 4 CH₃O, 36.3%.)

(B) *Physcion (emodin monomethyl ether, 4:5-dihydroxy-7-methoxy-2-methyl-anthraquinone)*. Physcion crystallizes when pure in long reddish-orange or brownish-orange needles, m.p. 203–4°, but appears to be dimorphic and tends, especially when slightly impure, to crystallize in large yellowish-orange lozenge-shaped plates. (Found: C, 67.61, 67.53; H, 4.20, 4.33; CH₃O, 10.6, 10.9%. Calc. for C₁₆H₁₂O₅, C, 67.57; H, 4.26; 1 CH₃O, 10.9%.) It is somewhat more soluble in organic solvents than is erythroglaucin. It is only slightly soluble in

light petroleum, somewhat more soluble in methanol, ethanol and ether, and readily crystallizes from ethyl acetate, glacial acetic acid, or a mixture of chloroform and ethanol. It gives a beautiful magenta colour in dilute solution in cold conc. H_2SO_4 , and since erythroglaucon gives a pure blue colour with this reagent it is possible to estimate roughly by this means the proportions of physcion and erythroglaucon present in mixtures of the two—a procedure which was used in their separation. Physcion gives a rose-pink solution with 2*N* NaOH from which a rose-pink precipitate slowly separates.

In order to establish the identity of the mould physcion a specimen of lichen physcion was prepared from an authentic sample of *Xanthoria parietina* (L.) (24 g.) kindly collected for us by Mr I. Mackenzie Lamb of the Natural History Museum, South Kensington, and by Dr W. Watson from a concrete post at Staplegrove, near Taunton, Somerset. The dried lichen was extracted with boiling light petroleum (B.P. 40–50°) and yielded 0.17 g. of crude product which on crystallization from glacial acetic acid gave pure physcion (0.1 g.), in yellowish-brown needles, M.P. 203–4°, not depressed on admixture with mould physcion. Since Raistrick *et al.* [1937] have already established, by direct comparison, the identity of mould physcion with Perkin & Hummel's [1894] emodin monomethyl ether from *Ventilago madraspatana* Gärtner, the identity of physcion from mould, lichen and climbing shrub follows.

Diacetylphyscion. A solution of mould physcion (0.02 g.) in acetic anhydride (1 ml.) containing a trace of conc. H_2SO_4 was boiled for a few minutes. The greenish-yellow solid obtained by pouring the solution into water was crystallized from ethanol. Fine yellow needles, M.P. 186–7°, unchanged on admixture with diacetylphyscion, M.P. 186–7°, prepared from lichen physcion. Perkin & Hummel [1894] give the M.P. as 185–6°.

(C) *Physcion anthranol A*, M.P. 260° (*emodinanthranol monomethyl ether A*). Physcion anthranol A was usually obtained from the mixture of mould colouring matters as colourless or very pale yellow needles which melt with decomposition at about 260°. (Found: C, 71.11, 71.01; H, 4.84, 4.89; CH_3O , 10.9, 11.3%. Calc. for $\text{C}_{16}\text{H}_{14}\text{O}_4$, C, 71.08; H, 5.22; CH_3O , 11.5%.) It is almost insoluble in benzene, xylene and alcohol and because of its slight solubility it can be fairly readily isolated from mixtures of the other mould colouring matters. It dissolves in cold conc. H_2SO_4 with a golden-yellow colour which changes in about an hour to dark green at room temperature and very rapidly on heating. It is only slightly soluble in aqueous NaOH giving a yellow solution which is slowly oxidized in air to give a rose-pink precipitate of the sodium salt of physcion. It agrees closely in its analysis, M.P., solubilities and colour reactions with those recorded by Perkin & Hummel [1894, pp. 935, 936] for the anthranol A which they isolated from *Ventilago madraspatana*. Like the latter it also gives physcion on oxidation but we have not succeeded in obtaining physcion anthranol A by the reduction of physcion.

Oxidation of physcion anthranol A to physcion. Physcion anthranol A (0.02 g.) was oxidized with chromic acid in an exactly similar way to that described later in section (D) for the oxidation of physcion anthranol B. The crude oxidation product (0.015 g.) was purified by sublimation in a high vacuum at 130–40° followed by crystallization from ethyl acetate. Yellowish brown needles, M.P. 203°, not depressed on admixture with lichen physcion from *Xanthoria parietina*.

(D) *Physcion anthranol B*, M.P. 181–2° (*emodinanthranol monomethyl ether B*). Physcion anthranol B is dimorphic, being usually obtained from the mixture of mould colouring matters as fine hair-like needles, but also as larger lozenge-

shaped plates especially when in association with physcion. When isolated from the mixed mould colouring matters it is usually pale pink in colour, probably because of the presence of traces of physcion from which it is very difficult to separate. After several crystallizations the pinkish colour still remains and even persists after fractional sublimation in a high vacuum at 130°. It melts at 181–2°. (Found: C, 70.76, 70.71; H, 5.08, 5.20; CH₃O, 11.0, 11.45 %. Calc. for C₁₆H₁₄O₄, C, 71.08; H, 5.22; 1 CH₃O, 11.5 %.) It is slightly more soluble in most organic solvents than is physcion but is somewhat less soluble in benzene from which it is best purified. With cold conc. H₂SO₄ it gives a golden-yellow colour which changes in about an hour to deep green. This change is brought about rapidly on warming. It dissolves slowly and with difficulty in aqueous NaOH to give an orange-red solution.

Reduction of physcion to physcion anthranol B. Physcion (0.97 g., i.e. 0.43 g. from *A. mongolicus* and 0.54 g. from *A. mutabilis*) was dissolved in boiling glacial acetic acid (150 ml.) and zinc dust (5 g.) was added in small portions until the reduction was complete. The excess of zinc was separated and the acetic acid solution, on cooling, deposited 0.48 g. of pale yellow needles, m.p. 181–2°. The acetic acid mother liquors, on dilution with 3 vol. of water, gave a further 0.30 g. of crude anthranol which was crystallized from benzene, giving 0.14 g., m.p. 180–2°. A final crystallization from benzene raised the m.p. to 182–3° not depressed on admixture with physcion anthranol B isolated from the mixed mould colouring matters. Hesse [1895], who also prepared this anthranol by the reduction of physcion isolated from *Xanthoria parietina*, gives the m.p. as 180–2° while Perkin & Hummel [1894] give the m.p. of their anthranol B from *Ventilago madraspatana* as 173°.

Oxidation of physcion anthranol B to physcion. To a boiling solution of physcion anthranol B from *A. oriolus* (0.05 g.) in glacial acetic acid (1 ml.) were added 2.5 ml. of a boiling 1 % solution of chromic acid in the same solvent. The mixture was boiled for 2 min. and was then diluted with an equal volume of water. On cooling, crude physcion (0.029 g.) separated and was crystallized from ethyl acetate giving 0.02 g. of yellowish brown needles, m.p. 203°, not depressed on admixture with lichen physcion from *Xanthoria parietina*. The two specimens also gave the same magenta colour reaction with cold conc. H₂SO₄. (Found: C, 67.42; H, 4.24 %. Calc. for C₁₆H₁₂O₅, C, 67.57; H, 4.26 %.)

SUMMARY

The colouring matters, extracted by light petroleum (b.p. 40–50°), and present in a number of species in the *Aspergillus glaucus* series, have been examined with special reference to the crude red colouring matters. Rubroglauclin, previously reported by Gould & Raistrick [1934] as occurring in certain species in the *A. glaucus* series, has now been shown to be a mixture of physcion (4:5-dihydroxy-7-methoxy-2-methylanthraquinone) and a hitherto undescribed colouring matter, erythroglauclin, which is the monomethyl ether of a tetrahydroxy-methylanthraquinone. In some of the species examined, erythroglauclin and physcion were accompanied by two reduction products of physcion, i.e. physcion anthranol A, m.p. 260° and physcion anthranol B, m.p. 181–2°. These anthranols were previously reported by Perkin & Hummel [1894] as being present, along with physcion, in the root-bark of the climbing shrub *Ventilago madraspatana*.

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CLIX. THE SULPHUR CONTENT OF FOODS

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MUCH information about the chemical composition of human foods has been collected in recent years, but there are still some wide gaps in our knowledge. Of all the systematic food tables which have been published, only those of Rosedale [1935], Sherman [1937] and possibly Schall [1938] give any original data about sulphur. Of these, Schall's figures are incomplete and sometimes uncertain, and Rosedale used a method [Morris & Rosedale, 1935] which other workers have condemned. Sherman's figures refer mostly to American foods. A search in the original literature for information about the amount of S in food-stuffs has confirmed the paucity of reliable systematic data. Schulz [1893; 1894] appears to have made the first investigation, which is now only of historic interest. Katz [1896] made a limited but very accurate study of muscular tissue. Balland [1907] and Sherman & Gettler [1912] made more extended investigations. More recently Kemmerer & Boutwell [1932] have determined S in 96 American food-stuffs. Their method seems to have been a reliable one, but unfortunately only one sample of each food was analysed. Stotz [1937] analysed 32 different plant and animal substances but he limited the value of his work by expressing the results only as percentages of the dry matter. Echevin & Crepin [1928], Frear [1930], Bertrand & Silberstein [1933, 1, 2; 1936; 1937], Schild & Jacob [1935], Painter & Franke [1936] and Balks & Wehrmann [1937] have determined S in various vegetables and plants. In recent years, since it has been shown that the addition of S or cystine to the diet of sheep improves both growth and wool production, many estimations of S in pasture grass and animal feeding stuffs have been made [Evans, 1931; Woodman & Evans, 1933; Warth & Krishnan, 1937].

Quite a number of authors have determined S in single substances. Thus S in milk has been studied by Steffen & Sullman [1931], Lachmann [1934], Kagi [1937] and Revol & Paccard [1937], and in eggs by Grossfield & Walter [1934] and Marlow & King [1936]. Friese [1929] and Sabalitschka [1931] made a detailed analysis of mushrooms, Le Matte *et al.* [1927] of animal organs used therapeutically, Morse [1929] of cranberries, Cleveland & Fellers [1932] of dates, and Wendt & Wilkinson [1931] and Steudel [1935] of lettuce and potato ash respectively. In each of these cases S was among the elements determined.

Of all these studies none has embraced the composition of foods as prepared for human consumption and few of them have yielded information of much value to those interested in human nutrition. For this reason it has been felt to be desirable to investigate and compare the relative values of a number of methods for estimating S in biological material, and then to make a systematic study of S in foods eaten in this country.

Methods, source of materials and sampling

Dried, mixed samples of nearly all the fruits, vegetables and nuts which had previously been analysed for other constituents by McCance *et al.* [1936] had been sealed up, and were available for analysis. The purchase and preparation of

these samples have already been fully described by these authors. S in certain vegetables must be determined on the fresh material (*vide infra*) and new samples of these foods were purchased from different shops, mixed and analysed without being dried. A number of food materials, notably the cereals, were being analysed concurrently for other constituents by McCance & Widdowson [1939]. The collection and sampling of these were carried out in a manner similar to that described for fruit and vegetables. Special purchases of meat and fish had to be made. At least three samples of every variety to be analysed were procured and equal portions of each were mixed and dried before any estimations were made.

S was determined in most instances by a hydrogenation method, though destructive oxidation of the organic matter with a nitric-perchloric acid mixture, followed by a gravimetric estimation of the resulting sulphates as BaSO_4 was used in some cases, particularly for the analysis of undried material. Both methods have been described in detail by Masters [1939].

Water was determined by drying to constant weight at 50° or 100° , and N by Kjeldahl's method [McCance *et al.* 1936] using a copper selenite catalyst.

Volatile S compounds

It may safely be said that S in most foods is present as cystine, methionine and stable organic and inorganic sulphates. Bertrand & Silberstein [1929], however, analysed a number of vegetables before and after drying them at 100° and found that onions lost S when so dried. They considered that this was due to the volatilization of essential oils containing S and suggested that the members of the Cruciferae and Liliaceae should be analysed for S in the wet state. Balks & Wehrmann [1937] have also pointed out that onions and horseradish lose S on being dried at 105° . They suggested that this was due to the volatility of allyl mustard oil, $\text{C}_3\text{H}_5\text{CNS}$, which these plants contain.

Blanck *et al.* [1937] extended these researches a little further. Beet leaves and horseradish were analysed fresh, after being dried at room temperature and at 80 – 100° . The leaves were found to lose no S, but the horseradish did so whichever way it was dried: as much as 33% disappeared during drying at 80 – 100° . 10% of the S in white cabbage was found to be volatile. Potatoes were held to lose no S during the process of being dried in the air.

Similar results were obtained by Rippel & Nabel [1937]. Chives, onions, leeks, horseradish, oatseed and asparagus were analysed fresh, and after being dried at 100° . The first four substances are said to contain mustard oil, though only leeks and horseradish were found to lose S on being dried. These losses, moreover, were reduced by a preliminary drying period in the air. No losses were detected when the plants were dried at room temperature.

Table I shows the effect of drying 28 representative foods at 50° for 48 hr. It will be observed that fruit, nuts, cereals, meat and beer lost no S. Fish lost small quantities, probably because it had not been purchased really fresh. Vegetables varied very much. Onions, horseradish, and mustard and cress lost large amounts of S, due no doubt to the presence in them of volatile essential oils. Watercress, savoy cabbage and brussels sprouts also lost some S. Some plants, e.g. cabbage, contain sulphur glucosides, the best known of which is sinigrin. This substance is hydrolysed by myrosin, dilute HCl or more slowly by boiling water, to glucose, KHSO_4 and allyl mustard oil [Simpson & Halliday, 1928]. The last is volatile and any which had been formed would be removed on drying at 50° . If, however, sinigrin is the source of the volatile S in cabbage, it is curious that boiled and raw cabbage lost the same amount of S when they were dried. Desiccation did not remove S in significant amounts from parsnips,

potatoes, peas or lentils. Vegetables, therefore, which it was thought might contain volatile, or potentially volatile S compounds, were analysed in the wet state, the others were analysed dry.

Table I. *Effect of drying on the S content of foods*

Substance	Determined after drying mg. S/100 g. or 100 ml. fresh material	Determined on fresh material mg. S/100 g. or 100 ml. fresh material
Cereals		
Pearl barley	124.5	121.5
Semolina	138.0	142.3
Drinks		
Beer	23.8	23.3
Fish		
Cod, raw	224.0	215.0
Plaice, raw	210.5	200.0
Meat		
Mutton chop, raw	237.0	233.0
Steak, raw	217.0	216.0
Fruit and nuts		
Apples	3.85	3.73
Bananas	14.4	14.9
Brazil nuts	339.0	337.0
Vegetables		
Brussels sprouts, boiled	97.5	89.2
Cabbage, savoy I, raw	204.0	174.0
Cabbage, savoy II, raw	115.0	96.7
Cabbage, savoy II, boiled	64.2	54.5
Cauliflower, boiled	76.5	79.7
Horseradish, raw	269.0	191.0
Leeks, boiled	90.0	89.0
Lentils, boiled	49.1	48.0
Lettuce, raw	13.7	15.5
Mustard and cress, raw	170.5	89.0
Onions, raw	53.7	36.7
Onions, spring, raw	70.0	47.5
Parsnips, raw	23.2	22.2
Peas, boiled	82.2	80.3
Potatoes, raw	36.2	34.2
Radishes, raw	46.7	42.7
Watercress, raw	61.6	52.8

Nitrogen: sulphur ratios

N had been determined by McCance *et al.* [1936] on all the mixed dried samples of fruits, vegetables and nuts, and an inspection of the N/S ratios has shown that they vary widely from one plant to another. This is probably to be attributed in most instances to differences in the composition of the proteins, but it may well be due to the variations in the inorganic constitution of those fruits with very little N or S in them. Table II gives the results for a few fruits and vegetables and shows the degree of variation. A study of 14 vegetables showed that the ratios were not altered appreciably by cooking. Four typical results are included in Table II. N was determined on all the mixed samples of meat and fish and the ratio was found to be relatively constant for any one class of material. The figures are given in Table IV. This constancy has a practical application for it seems unnecessary for dietary work to determine S in the muscular organs of a large number of animals and fish if N has already been

determined; S may be obtained quite accurately enough for all practical purposes from the average N/S ratio. Moreover, the ratio is unaffected by cooking (Table IV), and hence the S in cooked meat or fish may be calculated from the N of the cooked material and the N/S ratio of the raw material. The figures given by McCance & Widdowson [1939] for S in cooked meat and fish were obtained in this way.

Table II. *The N/S ratios of fruits, nuts and vegetables*

Substance	N/S	Substance	N/S	Substance	N/S
Apples, English, eating	5.1	Artichokes, Jerusalem, boiled	11.6	Beans, haricot, raw	20.5
Greengages	40.0	Peas, fresh	18.4	Beans, haricot, boiled	23.0
Oranges	14.0	Spring cabbage, boiled	6.7	Beans, scarlet runner, raw	12.8
Strawberries	7.5	Onions, raw	3.0	Beans, scarlet runner, boiled	12.7
Brazil nuts	7.8	Onions, boiled	3.8	Swedes, raw	4.6
Walnuts	19.3	Onions, fried	3.3	Swedes, boiled	4.6

Visceral organs, the elasmobranch fish and shellfish naturally fall into a different category. The ratio for elasmobranchs (e.g. dogfish) is probably high because of their relatively high non-protein N, whereas that for shellfish is always very much lower than the average figure for fish, possibly because the former are contaminated with sulphate from the sea water.

Results

These are given in Tables III and IV. The figures are expressed as mg./100 g. or 100 ml. of fresh matter (edible portion), and except where stated to the contrary, the results refer to raw materials. The percentage of water is not given in Table III, nor is the percentage of N, since neither was determined in this investigation. For this information reference can be made to McCance & Widdowson [1939]. McCance & Shipp [1933] or McCance *et al.* [1936] may be consulted for the scientific nomenclature of all the foodstuffs.

Discussion

In fruits and most vegetables the amount of S is low and very variable. The figures given were obtained on good mixed samples, but the individual scattering is large, and there may be wide differences between the amounts of S found in the same vegetable purchased on different occasions. This is no doubt partly due to the use of different manures or fertilizers [Davidson & Le Clerc, 1936; Bertrand & Silberstein, 1937]. Nevertheless, the S found in dried apricots and peaches is very much higher than an analysis of the fresh fruit would lead one to expect, and some of the S has probably been added in the form of SO_2 as a preservative [Leach & Winton, 1920]. The high figure for carrageen moss is probably due to large quantities of S in the form of the inorganic SO_4^{2-} radicle. This was the only material analysed in which the sulphate was the main acidic radicle.

The amount of S in meat is very much greater than that in vegetables and very much more constant for the different species.

A comparison of the present results with those of previous workers is rather unsatisfactory, owing to the inherent variability of so many of the foods and the uncertainty as to much of the sampling. Moreover, a study of the tables published by Schall [1938], Sherman [1937] and Kemmerer & Boutwell [1932] shows that there are large variations between the figures given for nearly every food analysed by these authors.

Table III. *The S content of foods other than meat and fish. Edible portions only have been analysed*

Name	S, mg./100 g.	Name	S, mg./100 g.
FRUITS, RAW		NUTS	
Apples, Empire, eating	3.7	Almonds	145.0
Apples, English, eating	7.6	Barcelonas	176.0
Apples, English, cooking	2.9	Brazils	293.0
Apricots	6.1	Chestnuts	29.4
Avocado pears	19.4	Cobs	74.5
Bananas	13.0	Coconuts	44.0
Blackberries	12.5	Coconut milk	23.8
Cherries, eating	6.8	Peanuts	377.0
Cherries, cooking	7.9	Walnuts	104.0
Cranberries	11.1	VEGETABLES	
Currants, black	33.1	Artichokes, globe, boiled	15.5
Currants, red	24.6	Artichokes, Jerusalem, boiled	21.6
Currants, white	23.6	Asparagus, boiled	46.6
Custard apples	26.7	Beans, baked	50.7
Damsons	6.4	Beans, broad, boiled	27.0
Figs, green	12.9	Beans, butter, raw	109.5
Gooseberries, green	15.9	Beans, butter, boiled	47.2
Gooseberries, ripe	13.5	Beans, French, boiled	8.3
Grapes, black	7.4	Beans, haricot, raw	166.5
Grapes, white	9.1	Beans, haricot, boiled	46.3
Grapefruit	5.1	Beans, runner, raw	14.1
Greengages	3.0	Beans, runner, boiled	9.5
Lemons, whole	12.3	Beetroot, boiled	22.1
Lemon juice	2.0	Broccoli tops, boiled	45.0
Loganberries	18.1	Brussels sprouts, boiled	77.8
Medlars	16.6	Cabbage, red, raw	68.0
Melons, cantaloupe	11.7	Cabbage, savoy, boiled	30.4
Melons, yellow	6.3	Cabbage, spring, boiled	26.7
Mulberries	8.8	Cabbage, winter, boiled	23.4
Nectarines	10.0	Carrageen moss, dried	5460.0
Oranges	9.0	Carrots, old, raw	6.9
Orange juice	4.6	Carrots, old, boiled	5.0
Passion fruit	18.7	Carrots, young, boiled	9.3
Peaches	5.7	Cauliflower, boiled	29.4
Pears, Empire, eating	5.6	Celeriac, boiled	12.8
Pears, English, eating	2.7	Celery, raw	14.9
Pears, English, cooking	3.4	Celery, boiled	8.3
Pineapple	2.6	Chicory, raw	12.7
Plums, Victoria, dessert	3.5	Cucumber, raw	11.0
Plums, cooking	4.6	Egg plant, raw	9.0
Pomegranate juice	4.2	Endive, raw	25.7
Quinces	5.2	Horseradish, raw	212.0
Raspberries	17.3	Leeks, boiled	48.9
Rhubarb	8.2	Lentils, raw	122.5
Strawberries	13.4	Lentils, boiled	37.3
Tangerines	10.3	Lettuce, raw	11.8
Tomatoes	10.7	Marrow, boiled	5.5
FRUITS, DRIED		Mushrooms, raw	33.8
Apricots	164.0	Mushrooms, fried	73.8
Currants	30.8	Mustard and cress, raw	170.0
Dates	51.0	Onions, raw	50.7
Figs	80.8	Onions, boiled	23.7
Peaches	240.0	Onions, fried	87.8
Prunes	18.5	Onions, spring, raw	50.0
Raisins	23.0	Parsnips, raw	16.5
Sultanas	44.3	Parsnips, boiled	14.6
TINNED FRUIT		Peas, fresh, raw	50.0
Tinned apricots	1.0	Peas, fresh, boiled	43.5
Tinned fruit salad	1.8	Peas, dried, raw	129.0
Tinned loganberries	3.0	Peas, dried, boiled	39.0
Tinned peaches	1.0	Peas, split, dried, raw	166.0
Tinned pears	1.3	Peas, split, dried, boiled	45.7
Tinned pineapple	2.7	Peas, tinned	43.9
Bottled olives (in brine)	35.6	Potatoes, old, raw	34.6

Table III (*cont.*)

Name	S, mg./100 g.	Name	S, mg./100 g.
VEGETABLES (<i>cont.</i>)		DAIRY PRODUCTS (<i>cont.</i>)	
Potatoes, old, boiled	22.2	Cream	33.0
Potatoes, old, chips	44.7	Egg yolk	164.5
Potatoes, old, roast	56.3	Egg white	182.5
Potatoes, new, boiled	24.3	Ice cream	30.6
Pumpkin, raw	9.5	Milk, fresh, whole	29.2
Radishes, raw	37.5	Milk, cond., unsweetened	75.0
Salsify, boiled	25.2	Milk, cond., sweetened	82.5
Seakale, boiled	52.0	Milk, cond., skimmed, sweetened	94.3
Spinach, boiled	86.5		
Spring greens, boiled	28.5	SWEETMEATS, JAMS, ETC.	
Swedes, raw	39.1	Chocolate, milk	67.0
Swedes, boiled	30.5	Chocolate, plain	32.0
Sweet potatoes, boiled	14.9	Cherries, glacé	21.0
Tomatoes, fried	9.2	Honey	0.8
Turnips, raw	22.1	Honeycomb	0.8
Turnips, boiled	21.2	Jam (edible seeds)	6.5
Turnip tops, boiled	39.0	Jam (stone fruits)	3.2
Watercress, raw	127.0	Jelly, packet	36.6
		Marmalade	2.1
CEREALS AND STARCH PRODUCTS		Mince-meat	28.4
All bran, Kellogg's	182.0	Sugar, Demerara	14.0
Arrowroot	1.6	Syrup, golden	53.8
Barley, pearl	117.0	Treacle, black	68.5
Biscuits:			
Cream crackers	77.8	BEVERAGES	
Digestive	72.0	Bournvita	243.0
Plain mixed	83.4	Bovril	362.0
Rusks	107.0	Cocoa	160.0
Sweet mixed	31.8	Coffee	110.0
Water	99.9	Malted milk, Horlick's	167.0
Bread:		Marmite	382.0
Currant	59.4	Ovaltine	183.0
Hovis	77.3	Oxo	321.0
Malt	114.5	Tea	177.0
White	54.5	Virol	82.9
Wholemeal	76.0	Beers:	
Buns, currant	73.4	Pale ale, draught	23.2
Cornflakes, Kellogg's	92.5	Pale ale, bottled	23.8
Cornflour	1.1	Mild ale, draught	20.4
Doughnuts	56.4	Mild ale, bottled	25.2
Dundee cake	55.0	Strong ale	34.1
Flour, white	108.5	Stout	23.1
Flour, wholemeal	123.5		
Force	105.0	CONDIMENTS	
Grapenuts	145.0	Curry powder	86.0
Macaroni	95.0	Ground ginger	145.0
Oatmeal	155.0	Mustard	1280.0
Post Toasties	83.0	Pepper	99.2
Rice	78.5	Salt: block	401.0
Ryvita	87.0	Table salt A	34.7
Sago	0.45	Table salt B	23.3
Semolina	91.8	Vinegar	18.6
Tapioca	3.5		
Vitaweat	93.2	FATS	
DAIRY PRODUCTS		Dripping	9.2
Butter	9.1	Lard	24.8
Cheese:		Margarine	12.1
Cheddar	230.0	Suet	20.0
Dutch	186.5		
Gorgonzola	177.0	SAUSAGES AND PASTES	
Gruyère	206.0	Beef sausage, fried	163.0
Packet	321.0	Black sausage	173.0
Parmesan	251.0	Breakfast sausage	78.5
St Ivel	186.0	Pork sausage, fried	95.0
Stilton	228.0	Fish paste	185.0
		Meat paste	131.0

Table IV. *The S content of flesh foods; edible portions only analysed*

Name	Water, g./100 g.	S, mg./100 g.	N/S	Name	Water, g./100 g.	S, mg./100 g.	N/S
MEATS				FISH (cont.)			
Beef, corned	55.6	222	17.0	Dabs	76.3	227	12.3
Beef steak	72.6	203	16.4	Dogfish*	72.4	132	17.4
Beef steak, fried	59.3	271	15.4	Eel	70.1	130	14.2
Beef steak, stewed	62.6	287	16.2	Fillet, smoked, boiled	75.9	249	13.1
Beef, topside	70.4	212	14.8	Haddock, fresh	80.2	226	12.0
Beef, topside, stewed	58.0	341	16.1	Haddock, smoked	73.4	221	14.7
Chicken, roast	47.2	232	14.9	Halibut, steamed	73.2	227	14.9
Duck, roast	42.6	395	13.8	Hake	80.3	164	16.1
Goose, roast	57.5	326	13.8	Herring	68.3	212	14.1
Ham, boiled	53.7	233	13.8	Herring, fried	55.3	270	13.5
Hare, roast	—	347	—	Herring's roe*	76.9	175	15.9
Lamb cutlet	65.3	166	15.1	Kippers	67.4	225	14.6
Mutton chop	65.6	197	15.5	Mackerel	70.1	162	16.3
Mutton, leg	52.4	164	15.4	Plaice	81.0	203	12.1
Pheasant, baked	51.5	302	13.7	Plaice, fried	68.0	246	12.6
Pork, leg	66.3	195	16.1	Salmon	72.0	192	16.9
Rabbit	75.0	169	17.8	Salmon, tinned	67.3	241	14.6
Turkey, roast	—	234	—	Sardines, tinned in oil	53.6	246	12.3
Veal	75.0	191	15.3	Smelts	76.8	168	14.2
Average			15.2	Sprats, smoked	62.3	222	15.2
ORGANS				Sole, Dover	78.4	233	12.5
Brain, sheep's	80.1	108	14.9	Sole, lemon	79.1	195	13.6
Heart, sheep's	75.4	176	14.1	Trout, rainbow	77.7	169	17.2
Kidney, sheep's	72.1	141	17.2	Turbot	80.0	188	14.1
Kidney, ox	76.0	154	17.5	Whiting	79.9	257	10.9
Liver, calves'	66.8	264	11.1	Whitebait	78.9	208	11.6
Liver, ox	69.0	263	11.8	Witch	79.1	181	12.6
Sweetbread	71.1	98	20.0	Average			13.8
Tripe	81.6	103	20.6	SHELLFISH†			
FISH				Cockles	65.7	286	5.6
Bloaters	69.3	234	12.2	Mussels	81.3	326	5.3
Brill	80.2	172	15.8	Mussels, boiled	79.0	262	7.9
Catfish	81.2	149	15.9	Prawns	66.3	335	9.9
Cod	82.6	171	14.1	Scallops	79.6	342	6.5
Cod, baked	71.3	256	13.7	Shrimps	59.4	339	11.2
Cod's roe	75.3	212	14.1	Whelks	75.9	401	6.6
				Winkles	75.1	265	6.5

* Not included in average.

† Analysed as purchased.

The present figures for fruit are of the same order as those given by the above workers, although Schall's results tend to be higher. It is not possible to compare the amounts of S found in vegetables with the results of the other authors, because all their analyses have been made on raw materials. The figures for raw pulses, however, are lower than those of Sherman. An average value only is given for the S in meat and fish by Sherman, but Katz and Schall have analysed the muscular tissues of a limited number of species, and the figures given in the present paper are of the same order and often agree closely with the results obtained by these workers.

The present figure for milk (29.2 mg. S/100 g.) agrees quite well with figures reported by other workers. Steffen & Sullman [1931] gave 30.52 mg., Revol & Paccard [1937] 27.44 mg., Schall [1938] 39.6 mg., Sherman [1937] 34 mg. and Kemmerer & Boutwell [1932] 28 mg.

Schall appears to have been the only one to have analysed different varieties of cheese, and the present authors' results agree with his findings, excepting where his figures are uncertain.

The yolk and white of eggs have been analysed separately, and the values obtained were 164 and 182 mg. S/100 g. respectively. These are in agreement with the findings of Kemmerer & Boutwell [1932], but are lower than those of Grossfield & Walter [1934]. The latter calculated the S in yolk and white from the most reliable figures available for the protein constituents of these substances. In this way they found yolk contained 201 mg. S/100 g. and white 216 mg. S/100 g. which agree well with the figures of 222 and 212 which they obtained on analysis. Schall, however, gives 176 and 276 mg. S/100 g. for yolk and white respectively, and Sherman gives an average figure of 204 mg. 100 g. for S in the whole egg.

Table V. *The amount of S found in cooked dishes, as determined by calculation and analysis*

	S calculated mg./100 g.	S found mg./100 g.
Apple charlotte	12.4	14.0
Fish pie	105.0	108.5
Macaroni cheese	68.0	64.0
Queen cakes	81.0	72.0

As a general test of the reliability and applicability of the figures given in Tables III and IV, four cooked dishes were prepared as they would be normally for human consumption. The ingredients were not "mixed samples". The recipes were known and from these and the change of weight on cooking the S in the cooked dish was calculated, using the data in Tables III and IV. The food was also analysed and it will be seen from Table V that the amount of S found on analysis agrees satisfactorily with the calculated figure.

SUMMARY

1. Drying introduces errors into the determination of S in certain vegetables owing to the presence in them of volatile or potentially volatile S compounds.
2. Figures are given for the total S found in about 300 different foodstuffs.
3. The N/S ratio has been shown to be relatively constant for all muscular organs. The average figure for meat is 15.3 and for fish, 13.8. It is lower than this in all shellfish, and varies from one visceral organ to another. The ratio varies widely in different plants so that generalization about them is not justified.
4. The results have been compared very briefly with those of previous authors.

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CLX. THE DETERMINATION OF SULPHUR IN BIOLOGICAL MATERIAL

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THE first comprehensive paper on the determination of sulphur in biological material was published by Barlow [1904], who reviewed twelve methods which were then in common use and showed that all tended to give low results owing to loss of sulphur by volatilization. He described his own method in which organic material was burnt in a combustion tube and all the sulphur caught as SO_3 in a quartz-sodium carbonate column. This procedure appears to have been very accurate though a little cumbersome for routine use.

The destruction of organic matter without loss of sulphur always presents difficulty because sulphur and many of its compounds are very volatile. It may be done by fusion of the substance with an alkaline oxidizing mixture, by "wet" ashing or by hydrogenation.

Fusion methods

Early workers tried to prevent loss of sulphur during dry ashing by addition of alkaline salts, e.g. $\text{Ba}(\text{OH})_2$, CaCO_3 , Na_2CO_3 [see Barlow, 1904]. More recent investigators have fused the substance with an alkali such as Na_2CO_3 , NaOH , or Na_2O_2 . Of these, Na_2O_2 appears to have been the most successful and is still in use to-day.

Folin [1905] described the determination of total sulphur in urine by sodium peroxide fusion and Sherman [1902] a similar method for foods. These were the earliest applications of the method to biological material. Since its original introduction, many modifications of this procedure have been suggested, though the most serious difficulty encountered, i.e. the frequency with which explosions take place, has not yet been satisfactorily overcome, except by the use of a bomb [Parr, 1908]. This, however, involves the use of special and expensive apparatus [Parr Instrument Co., Booklets 115, 116, 117].

The fusion method has been adopted as the official method of the Association of Official Agricultural Chemists [1935] and the technique recommended by it has been found by the author to give accurate results, but to be unsuitable for the routine determination of sulphur. To prevent explosion or ignition of the fusion mixture, which ruins the estimation, constant care and attention have to be given to each individual fusion, every one of which has to be done separately and takes at least 1 hr.

Wet ash methods

A number of workers have attempted to apply the copper nitrate oxidation method of Benedict [1909], originally introduced for determination of total S in urine, to general biological analysis. Wolf & Osterberg [1910] and Halverson [1919] found preliminary treatment with HNO_3 to be necessary. Waelsch & Klepetar [1932] found that adjustment of the pH of the solution to about 7.2 by the addition of Na_2CO_3 gave better results, though Rutender & Andrews [1937] showed that the results varied according to the amount of Na_2CO_3 added. For

the determination of sulphur in pure organic compounds Hoffman & Gortner [1923] showed that the substance must be in solution so that finely divided particles come into contact with $\text{Cu}(\text{NO}_3)_2$.

Frear [1930] adapted the Benedict-Denis method for use on plants with apparent success but in the author's hands this method was found unsatisfactory. Painter & Franke [1936] have experienced similar difficulties which they explained by the failure of the Benedict-Denis mixture to oxidize methionine-sulphur quantitatively. This failure is further emphasized by work of Waelsch & Klepetar [1932], who found only 0.25 % S in casein analysed by this method, whilst the accepted figure for casein-S is 0.8 % [Sherman, 1937]; according to Baernstein [1936], 83.4 % of the total S of purified casein is present as methionine. Painter & Franke also obtained lower figures for casein-S by the Benedict-Denis method than by the Parr bomb method; the author's results (Table VI) confirm the findings of these workers. For the analysis of many biological substances, therefore, the Benedict-Denis method is of little use.

The use of HNO_3 for the destruction of organic matter. Many methods of "wet" ashing have been introduced in recent years, all of which involve the use of HNO_3 generally in conjunction with another oxidizing agent. Thus Stockholm & Koch [1923] used a "wet" ash technique employing H_2O_2 and HNO_3 in preference to a dry fusion. Blanck *et al.* [1937] compared results by ashing, with and without addition of alkali, with three wet ash techniques (digestion of material with HNO_3 and KClO_3 , with aqua regia and KClO_3 or with NaOH , H_2O_2 and Br); ashing without addition of alkali always resulted in losses of S. The other methods all yielded similar values for S, but digestion with HNO_3 and KClO_3 was found to be the most satisfactory as it was quicker and simpler. Bertrand & Silberstein [1929] destroyed the organic matter and oxidized the S in plants by heating them with HNO_3 , followed by an alkaline fusion; a similar process was used by Rippel & Nabel [1937] and by Blanck & Sachse [1938] and was found by the latter authors to be more reliable than five other wet ash methods investigated for the analysis of taurine, cystine and allyl isothiocyanate. Cherbuliez & Meyer [1933] mixed the material with an equal weight of NaNO_3 and 30 ml. fuming HNO_3 for the oxidation, whilst Echevin & Crépin [1928] mixed the substance to be analysed with $\text{Mg}(\text{NO}_3)_2$ and subsequently heated it with HNO_3 . Revol & Ferrand [1935] used a mixture of HNO_3 and perhydrol and passed the escaping fumes into a NaOBr solution to guard against any loss of S. Warth & Krishnan [1935] gave the material (urine, faeces and feeding stuffs) a preliminary oxidation with HNO_3 , and followed this by fusing the evaporated filtrate with NaOH in a silver basin. Stotz [1937] found that fusion of the material with peroxide with or without preliminary digestion with HNO_3 might fail to convert S into SO_4^{2-} and stated that ashing in presence of KOH and KMnO_4 effected complete oxidation.

The nitric-perchloric acid method. This method was first introduced by Kahane [1927] for the determination of S in rubber; in the same year Le Matte *et al.* [1927, 1, 2] used it for the analysis of animal tissues. Wolesensky [1928] also employed it for estimating S in rubber, and it was later used on biological material by Toepfer & Boutwell [1930], who preferred it for large samples to the Parr bomb. Kemmerer & Boutwell [1932] used the method for destroying organic material before estimating S in a large number of foodstuffs. Giesecking *et al.* [1935] used HNO_3 and HClO_4 for the destruction of organic matter in plants.

Balks & Wehrmann [1937], however, digested the material with HNO_3 and HClO_4 only after it had been given a preliminary heating with 10 % aqueous NaOH and treated with H_2O_2 , since they found the method was only satis-

factory for taurine and allyl isothiocyanate under these conditions; these substances, however, are not widely distributed in biological material. Kăgi [1937] used this mixture of acids for estimating S in milk.

Present investigation of the nitric-perchloric method

Method of digestion adopted. The material to be analysed (1–5 g.) was weighed out, brushed into a 100 ml. long-necked Kjeldahl flask and 5 ml. of conc. HNO_3 added. A glass bulb was placed in the neck of the flask to prevent loss by sputtering. The mixture was kept on a sand bath at a low temperature till fumes of HNO_3 no longer came off. Care had to be taken during this initial reaction to prevent the contents frothing out of the flask. When effervescence had ceased a further 3 ml. of HNO_3 were introduced, and as soon as solution was complete, 2 ml. of HClO_4 were added and the temperature raised. Additions of HNO_3 and HClO_4 were made at intervals until a clear colourless solution resulted, usually after some 16 hr. By this time all the HNO_3 had been boiled off and the solution contained HClO_4 and salts only. The later stages of the digestion sometimes had to be taken more slowly when analysing fatty materials to minimize effervescence and bumping.

The solution was then heated on the sand bath for at least 12 hr., more HClO_4 being added as necessary. Finally, it was allowed to evaporate to a volume of 2 ml. When the residue was allowed to cool, a small white precipitate occasionally formed which sometimes dissolved on addition of water. In this case it was assumed to be KClO_4 . At other times it remained insoluble, when it was assumed to be SiO_2 and was filtered off. Many workers [Kemmerer & Boutwell, 1932; Toepfer & Boutwell, 1930; Wolesensky, 1928; Kahane & Kahane, 1934] have evaporated the solution to dryness with HCl after adding NaCl to fix the free H_2SO_4 in order to remove the excess HNO_3 and HClO_4 . HNO_3 , when present, interferes with the precipitation of BaSO_4 [Treadwell & Hall, 1935]; the author found this refinement unnecessary as all the HNO_3 had already been boiled off, and only 1 or 2 ml. of HClO_4 remained, which did not appear to interfere in any way with the precipitation of BaSO_4 . The results shown in Table I demonstrate this point.

Table I. *Analysis of substances containing known amounts of S by nitric-perchloric method*

Substance	Amount taken mg.	S calc. mg.	S found mg.	% recovery
Na_2SO_4	4.42	1.0	1.03	103.0
			0.96	96.6
,,	8.84	2.0	2.03	101.5
			1.95	97.5
,,	13.26	3.0	3.00	100.0
			3.03	101.0
,,	17.7	4.0	4.00	100.0
			4.00	100.0
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	6.08	1.57	1.61	102.6
Cystine	50.00	13.385	13.46	100.8
Methionine	50.00	10.75	10.90	100.8

N.B. Estimations of N on the samples of cystine and methionine used for analysis showed them to be 96 and 93 % pure respectively. Allowance was made for this in the above calculations.

Explosions. Occasionally there have been explosions during the wet digestions. Kahane [1937] described a similar experience and stated that the action of

HClO_4 on organic substances may become explosive in the absence of a diluent such as H_2SO_4 or excess HClO_4 . Kägi [1937] also found excess of HClO_4 to be necessary because the mixture exploded if the solution was evaporated to dryness before all the organic matter had been destroyed. The author, however, found that the digestion mixtures were liable to explode both before and after the addition of HClO_4 ; but that they did not do so if the preliminary stages were taken slowly. These explosions may be due to the formation of nitro-compounds.

Estimation of sulphate. The solution in the Kjeldahl flask was washed out into a 500 ml. beaker, and the sulphates resulting from the oxidation were estimated gravimetrically as BaSO_4 in the usual way [Treadwell & Hall, 1935].

Effect of iron on the precipitation of BaSO_4 . Tervalent metals may interfere with the precipitation of BaSO_4 [Treadwell & Hall, 1935]. In consequence some authors [Evans, 1931; Cherbuliez & Meyer, 1933] remove iron before precipitating the sulphate.

Varying amounts of FeCl_3 were added to solutions of Na_2SO_4 containing 4 mg. S. The sulphate was estimated gravimetrically and the results are shown in Table II.

Table II. *Effect of iron on BaSO_4 precipitates*

Amount of Fe added mg.	Theoretical amount BaSO_4 mg.	Amount BaSO_4 recovered mg.	Difference mg.
0.3	29.0	29.3	+ 0.3
0.2	29.0	28.9	- 0.1
0.1	29.0	28.0	- 1.0
0.05	29.0	28.8	- 0.2
0.01	29.0	28.5	- 0.5
0.005	29.0	28.6	- 0.4

} Filter pad brown

} Filter pad uncoloured

When the solution contained 0.05 mg. Fe or more the asbestos filter pad in the crucible was coloured brown. In the many estimations performed by this method coloration of the filter never occurred. Hence it would appear that in no case had there been more than 0.05 mg. Fe present in the solution. Table II shows that such amounts do not affect the result beyond the limits of experimental error (± 0.5 mg. of BaSO_4).

Analysis of substances containing known amounts of S. Analysis of standard solutions of Na_2SO_4 , $\text{Na}_2\text{S}_2\text{O}_3$ and of cystine and methionine showed the method to give results accurate to within 3% (Table I) and greater accuracy was obtained when larger quantities were analysed, because the precipitates could be weighed more precisely.

Determination of S in casein. The determination of S in casein has been studied and its oxidation has been found to present more difficulty than that of

Table III. *Effect of prolonged digestion on the determination of S in casein*

Sample	Duration of heating after solution became clear hr.	% of S found
A	0	0.700
		0.645
B	1	0.716
		0.722
C	6	0.745
		0.724
D	12	0.750
		0.770
E	24	0.750
		0.770

cystine or similar compounds; at first low figures were obtained and good duplicates were rare. However, if boiling was continued after the solution in the Kjeldahl flask had become clear, the method gave higher and more consistent results (Table III); heating for 12 hr. appeared to be sufficient. The figures are still lower than those quoted earlier, but nevertheless are in good agreement with those obtained by the fusion method (Table VI).

Loss of S during oxidation. To avoid the possibility of loss of S by volatilization during oxidation, bombs have frequently been used [Sherman, 1902; Parr, 1908; Evans, 1931; Painter & Franke, 1936; Godden, 1937]. A bomb, however, is tedious to use when many estimations have to be made. Revol & Ferrand [1935] prevented loss by distilling into bromine in alkali, which converted SO_2 into SO_3 . Kahane & Kahane [1934] estimated the S in pure organic substances with a mixture of I_2O_5 , HClO_4 and HNO_3 , trapping the escaping gases in a solution of I_2O_5 to prevent any loss of H_2S or SO_2 .

Wolesensky [1928], who used the nitric-perchloric method for the estimation of S in rubber, stated that he obtained good recoveries and hence no S appeared to be lost as spray. If, however, the digestion mixture was strongly heated so that oxidation took only 7–8 min., as originally recommended by Kahane, much S was lost. The present author found that when the substance was oxidized slowly, no S was detectable in the escaping gases when these were drawn through a trap which contained distilled water. (Glass joints were used in this apparatus because the fumes of HNO_3 attacked the rubber bungs, resulting in S contamination.) Any gases containing S escaped into an atmosphere of HNO_3 and HClO_4 ; hence it was not considered necessary to add any further oxidizing agent to the trap. This was checked by analysing one substance twice, the first time with distilled water, and then with NaOBr in the trap. The results were compared and showed no difference.

The contents of the flask and trap were washed out separately, and BaCl_2 added to each. Eleven different substances were analysed in duplicate in this way. In eight of them no precipitate was observed when a solution of BaCl_2 was added to the washings from the trap. Where a precipitate did occur it was confined to one of a pair of duplicates, and was in any case almost imponderable (i.e. less than 0.2 mg. S). The S in the samples taken for analysis varied from 1.5 to 13.6 mg.

Casein was the only substance which persistently gave a precipitate in the trap. The total weight of BaSO_4 obtained from the washings of flask and trap was the same, however, as that obtained from the flask when no trap was attached. This phenomenon was thought to be explained by the fact that the gases containing volatile S compounds would normally be prevented from escaping by the refluxing action of the long-necked flask. When the trap was attached to the flask and suction employed this action was lost and the gases were drawn over into the water. It was concluded that a trap was not necessary.

Recovery of added S. S in the form of Na_2SO_4 , Na_2SO_3 and cystine was added to a number of foodstuffs. A study of Table IV shows all the recoveries to be between 95 and 105%, the majority being very much closer to 100%. As often only 10–30 mg. BaSO_4 were obtained from the material used, results of greater accuracy could not be expected.

Table IV. *Recoveries of added S by the nitric-perchloric method*

Substance	Amount of S in sample taken mg.	S compound added	S added mg.	S recovered mg.	% recovery
Butter beans	1.17	Na_2SO_4	2.00	2.02 2.00	101.0 100.0
Chocolate	1.50	Na_2SO_4	2.00	2.08 2.03	104.0 101.5
Almonds	3.10	$\text{Na}_2\text{S}_2\text{O}_3$	2.99	2.87 2.99	96.0 100.0
Biscuits (mixed plain)	2.50	$\text{Na}_2\text{S}_2\text{O}_3$	2.99 1.51	3.00 1.48	100.5 98.1
Malted milk	3.24	$\text{Na}_2\text{S}_2\text{O}_3$	2.99	2.97	99.5
Sausages	2.90	$\text{Na}_2\text{S}_2\text{O}_3$	2.99	3.00 2.85	100.5 95.5
Barcelona nuts	3.75	Cystine	5.10	5.16 5.05	101.1 99.4
Peas (split, dry)	2.00	Cystine	5.10	5.19 5.25	101.5 102.8
Oranges	1.35	Cystine	5.10	5.08 5.01	99.5 98.0
Sago	0.20	Cystine	5.10	5.19 5.44	101.5 105.2
Salsify	2.68	Cystine	5.10	4.88 5.18	95.5 101.5
Vitaweat	3.05	Cystine	5.10	5.09 5.07	99.8 99.5

Conclusions

This procedure appears to have many advantages. The ashing can be done in a pyrex Kjeldahl flask, and therefore large quantities of materials with low S content can be taken. When possible, enough material was taken to give at least 50 mg. of BaSO_4 , as the balance used weighed to 0.1 mg. The oxidation requires very little attention, and so many estimations can be made simultaneously.

Hydrogenation method

All the methods so far described depend on the *oxidation* of S to sulphate. ter Meulen [1922] introduced a method of estimation for use with organic compounds, depending on the *reduction* of S to H_2S , which was estimated iodimetrically; as yet this method has not been much used. A very similar method is described by Kubota & Hanai [1928].

ter Meulen only applied the method to pure organic substances, oils and coal gas. There appeared to be no reason why it should not be applied to biological material such as foods, urine and faeces. Investigation showed this to be possible, and the technique finally developed follows.

Reagents. A cylinder of hydrogen (a Kipp's apparatus is unsatisfactory).

Platinized asbestos.

B.D.H. A.R. pellet NaOH. 2 pellets = 0.25 g.

A strong solution of NaOH.

Solution A. 2 % CdCl_2 .

Solution B. 1 % CdCl_2 in 20 % acetic acid.

20 % acetic acid.

N/100 $\text{Na}_2\text{S}_2\text{O}_3$ standardized against N/100 KMnO_4 .

N/100 I_2 dissolved in KI, standardized against N/100 $\text{Na}_2\text{S}_2\text{O}_3$.

Apparatus. The apparatus is shown in Fig. 1. The five-litre jar *A* contains hydrogen, and a steady stream of gas can be procured at any desired pace by running water into the bottle from a tap.

The wash bottle *B* contains a strong solution of NaOH. A blank estimation showed no further purification of the hydrogen to be necessary. The glass connexion between the wash bottle and the combustion tube is drawn out to a fine jet to prevent the gas from blowing back.

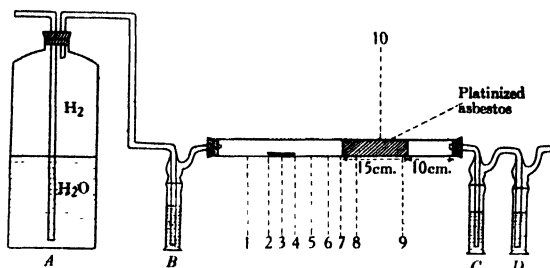


Fig. 1. Apparatus employed for the determination of S by hydrogenation.

The combustion tube is made of quartz, and transparent or opaque tubing can be used with equal success. The latter is to be recommended, for it is cheaper, and also frequent heatings soon render a transparent tube opaque. The tube employed is of 10 mm. diameter, and 60 cm. long. A small diameter is most successful, as it is easier to displace all the gas.

The combustion tube is loosely packed with platinized asbestos for a length of 15 cm. as shown in the diagram; corks are used in preference to rubber bungs since contamination from the former is less likely.

The mixture of gases from the heated combustion tube is passed through two wash bottles, *C* and *D*, each of which contains two pellets of NaOH and 1 ml. of solution A.

Kubota & Hanai used a tube with wire coiled round it so that all portions of the tube were heated equally. In the present investigation the tube was heated by means of Bunsen burners with spreaders attached.

Procedure for hydrogenation. (1) The substance was introduced into the quartz tube in a boat, and the corks inserted and waxed over.

(2) Hydrogen was passed through the tube rapidly, until sufficient time had elapsed (i.e. 5-10 min.) for all the air to have been displaced. The rate of flow of hydrogen was then adjusted to one bubble per sec., which experiment had shown to be most satisfactory for H_2S formation.

(3) The platinized asbestos was heated to a dull red heat by Bunsen burners placed in positions 8, 9 and 10 (Fig. 1). Meanwhile, the tube was gradually heated from positions 1 to 7 (Fig. 1) by two more Bunsen burners. One without a spreader was placed in position 1, and then moved towards position 7, at approximately 5 min. intervals, so that the total time of heating was about 35 min. As the first burner was moved along the tube, a second one, with spreader, was introduced behind it. The region under the boat was heated very gradually. In each position the tube was heated, gently at first, and then the intensity of the flame gradually increased to its maximum. This technique was adopted so that the substance was volatilized slowly.

Position 7 having been reached, heating was continued for another 15 min. to ensure complete hydrogenation.

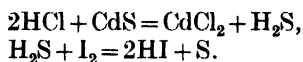
(4) The Bunsen burner in position 9 was moved along to the right (Fig. 1). Experiment showed that some substances would volatilize and condense in this part of the tube, as often a considerable quantity of H_2S came over when this portion was heated.

(5) The rate of flow of hydrogen was gradually increased to make sure that all the H_2S present was displaced. When H_2S had ceased to come over (*vide infra*) the hydrogenation was ended and the CdS estimated.

During hydrogenation carbon is deposited on the catalyst and walls of the tubes. The catalyst is thereby poisoned and, if the tube is not cleaned, low results are obtained, as H_2S is absorbed by the carbon. The latter is removed and the tube made ready for another analysis by displacing the hydrogen and then heating to redness in a current of air. ter Meulen [1922] recommended that substances that charred excessively should be mixed with platinum black before hydrogenation to prevent absorption of H_2S by carbon. The tube should also be cleaned by rinsing it with HCl after it has been used for a number of estimations.

Estimation of H_2S . When the NaOH and CdCl_2 were mixed in the wash bottles, a white precipitate of $\text{Cd}(\text{OH})_2$ was thrown down. As soon as H_2S commenced to come over yellow CdS was formed. Since no yellow precipitate ever appeared in the second wash bottle, it was concluded that all the H_2S was absorbed in the first one, and the second bottle was later discarded. This colour change was also used to indicate the end point of the hydrogenation. The wash bottle in which H_2S was being collected was replaced by a fresh one. If the precipitate in this jar remained white, heating was terminated.

The contents of the wash bottle were transferred to a 50 ml. centrifuge tube, and 2.5 ml. of solution B added. This either dissolved any residual $\text{Cd}(\text{OH})_2$, or, on the other hand, ensured the complete precipitation of S as CdS should all the $\text{Cd}(\text{OH})_2$ have been used up and some H_2S be held by the NaOH. After centrifuging for 5 min. the supernatant fluid was decanted and the precipitate washed with 10 ml. of 20 % acetic acid. The tube was again centrifuged, and the supernatant fluid removed. A known excess of $N/100 \text{ I}_2$, varying with the size of the precipitate, was added, and the whole acidified with conc. HCl, until solution was complete. (It was essential to add an excess of HCl because CdS is insoluble in acid weaker than 1.3*N*.) The reaction proceeds according to the equations:



The excess I_2 was titrated with $N/100 \text{ Na}_2\text{S}_2\text{O}_3$. The amount of S was then calculated from the equation

$$1 \text{ ml. } N/100 \text{ I}_2 = 0.16 \text{ mg. S.}$$

Very accurate and reliable results were obtained by this method (Table VI).

Discussion of hydrogenation method

The method, as given above, differs somewhat from ter Meulen's, although the principle remains the same.

1. *Apparatus.* The relative merits of hard glass and quartz tubing for the above estimation have been discussed by other workers. ter Meulen originally recommended quartz tubing, and platinized asbestos as catalyst, but later [1931] he stated that as good results had been obtained by using pure in lieu of platinized asbestos. Gauthier [1935] obtained low values when he employed a hard glass tube and pure asbestos, and said that satisfactory results were only

obtained if pumice stone was used as a catalyst. ter Meulen [1935] replied to this by pointing out that if pure asbestos was used, the tube had to be heated to at least 1000° and must therefore be of quartz. The present author found hard glass tubing unsatisfactory, both with pure and platinized asbestos. In both cases very low results were obtained, owing, no doubt, to the fact that the tubing could not be heated strongly enough without its beginning to collapse. Quartz tubing and platinized asbestos gave the best results. With pure asbestos results were not so reliable, in some cases being slightly too low; this was probably due to the fact that the tube was heated by Bunsen burners, and not by a furnace. In consequence, a sufficiently high temperature was not reached.

2. *Estimation of H_2S .* ter Meulen [1922], in his original method, absorbed the H_2S in NaOH. At the end of the hydrogenation, the NaOH was washed into a known excess of I_2 solution and acidified, and the S was estimated by a back titration of the I_2 with $Na_2S_2O_3$.

The author, used this technique and obtained very satisfactory results with cystine and methionine. However, when more complex substances such as casein or foods were analysed in the same way, the results were irregular and high. No permanent end-point could be obtained and it was in any case difficult to discern, owing to the solution turning a greenish colour. For consistent and accurate results, H_2S has to be separated from any compound which might interfere with its estimation. This was achieved by precipitating it as insoluble CdS.

ter Meulen [1934] showed that when a platinum spiral was used as a catalyst and N was present in the compound analysed, $(CN)_2$ was formed, and interfered with the I_2 titration. HCN was liberated on acidification of the NaOH solution, and this absorbed I_2 , thereby giving high results. He overcame the difficulty in a manner very similar to that of the author, though this was not known at the time when the present method was devised.

Unsaturated hydrocarbons would also interfere, if present, though this was considered unlikely. No doubt the unreliable results obtained by the present author for casein etc. were explained by the formation of $(CN)_2$ in the presence of platinized asbestos, though it is curious that a similar interference did not upset the estimation of S in cystine and methionine.

In all estimations $N/100 I_2$ and $N/100 Na_2S_2O_3$ solutions were used, which enabled amounts of S as low as 0.2 mg. to be estimated. ter Meulen [1922] has described a colorimetric micro-method for estimating quantities as low as 0.05 mg. S; the H_2S is converted into PbS and the colour compared with a standard preparation of PbS made from NaS. The chief difficulty of this method is to prevent the standard Na_2S solution from becoming oxidized, though Giberton [1933] has described a very simple means of standardizing the Na_2S before use.

3. *Interference by metals.* In his original paper ter Meulen [1922] stated that when alkali salts were present, some S remained behind, attached to the metal. This S could be recovered by fusion of the residue in the boat with borax. Gauthier [1935] used this technique, but only recovered half the S when analysing alkali sulphates, and recommended instead that after a preliminary hydrogenation, the boat should be withdrawn, a drop of HCl added, the boat replaced and heating continued.

The author intended to use this method for the analysis of substances which contained alkali salts (urine, foods etc.) and the matter was further investigated. Three foods and a specimen of urine were analysed with and without the addition of borax and HCl. Table V shows that the results were often lower

and more inconsistent when borax was added, than those obtained when no additions were made. With HCl the results tended to be very slightly higher, but not significantly so. From this it would appear that the addition of either borax or HCl to the residue in the boat is unnecessary, and that the method may be used as it stands for the analysis of biological material.

Table V. *Effect of adding HCl or borax to substance in the boat*

Substance	mg. S/100 g. or 100 ml. Found after addition of		
	Nil	Borax	HCl
Meat, dried	752.5	660.0	757.5
Pearl barley, A	108.0	111.0	109.7
Peas, split, dried	166.0	156.0	170.5
Urine	46.6	34.7	46.1

Comparison of investigated methods

Table VI gives a comparison of results obtained by all four methods investigated. Two points are at once apparent:

(1) The Benedict-Denis technique fails entirely with methionine and casein, and cannot therefore be recommended as a general method.

(2) The agreement between the remaining two oxidation methods and the hydrogenation method is, on the whole, good.

Table VI. *S determinations by four different methods*

Substance	% S by the various methods				Theoretical %
	Benedict-Denis	Sodium peroxide fusion	Nitric-perchloric oxidation	Hydrogenation	
Cystine	27.00	26.55	26.90	26.80	26.67
Methionine	9.10	21.70	21.80	21.60	21.50
Casein	0.47	0.77	0.76	0.77	—
Biscuit, dried	—	0.102	0.102	0.117	—
Meat, dried	—	0.625	0.630	0.600	—
Pearl barley, B	—	0.135	0.124	0.117	—
Peas, dried	—	0.178	0.186	0.166	—
Sausages	—	0.157	0.150	0.165	—
Urine (24 hr. specimen) A	—	—	0.047	0.047	—
Urine (24 hr. specimen) B	—	—	0.068	0.071	—

All three methods give figures in good agreement with the theoretical S values of cystine and methionine. With casein, and also with other foods, agreement is close, though greater variations are found between the figures obtained for these substances than for the simpler compounds.

Disadvantage of the hydrogenation method. The method has one minor disadvantage. It is not easy to run several estimations concurrently. The author found it possible, however, to carry out two estimations at the same time by placing the combustion tubes side by side and heating them both with the same set of Bunsen burners.

Advantages of the hydrogenation method. The hydrogenation method has many advantages when compared with oxidation methods.

(1) The final stage is a titration. This enables smaller quantities of S to be estimated more accurately than can be done by the oxidation-gravimetric methods. This in turn means that smaller quantities can be taken for analysis.

(2) The method is considerably quicker than any oxidation process. A complete estimation can be done in $2-2\frac{1}{2}$ hr., whereas all the other methods take at least 24 hr. and often longer.

(3) There is no risk of losing S by volatilization.

SUMMARY

1. Four methods of estimating sulphur in biological material have been investigated. Three of these methods, i.e. the Benedict-Denis, the sodium peroxide fusion and the nitric-perchloric methods, were oxidation processes. The fourth involved hydrogenation.

2. The sodium peroxide fusion method gave accurate results, but frequent explosions made it unsatisfactory for routine use.

3. The Benedict-Denis method was found to be unreliable, probably because of its failure to determine methionine-sulphur accurately.

4. The nitric-perchloric method, in which destruction of the organic material by a mixture of HNO_3 and HClO_4 was followed by a gravimetric estimation of the resultant sulphates, appeared to be reliable. Large quantities of material could if necessary be taken, and the oxidation required very little attention, so that many estimations could be made simultaneously.

5. The hydrogenation method was applied to biological material, and was found satisfactory after certain modifications had been made. The results by this method compared favourably with those given by the fusion and nitric-perchloric methods. It was found to be a quicker and more sensitive method than any oxidation process investigated, and seemed to be the best available for the routine determination of sulphur.

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CLXI. THE ISOMERIZATION OF CAROTENES

III. RECONSIDERATION OF THE CHANGE β -CAROTENE TO $\psi\alpha$ -CAROTENE

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EARLIER experiments on the chromatographic adsorption of β -carotene on Tswett columns of alumina or calcium hydroxide have shown that this pigment readily undergoes change into another carotenoid having a distinctly different absorption spectrum [Gillam & El Ridi, 1935]. Later work resulted in the isolation of a new crystalline carotenoid exhibiting an absorption spectrum different from that of β -carotene but identical with that of α -carotene. Further investigation and analysis showed this to be a carotene isomeride differing from α -carotene in certain properties and it was therefore named *pseudo- α -carotene* ($\psi\alpha$ -carotene) [Gillam & El Ridi, 1936]. A similar study of the adsorption of α -carotene indicated that this pigment could also be changed into yet another new entity (differentiated by means of its absorption spectrum) which was provisionally called neocarotene. On crystallization it was found that the absorption spectrum had reverted to that of the parent α -carotene. The properties of the crystals were however quite different from those of either α -carotene or $\psi\alpha$ -carotene, being in fact those of another new carotenoid which has been named neo- α -carotene, although the absorption spectra of all three are identical [Gillam *et al.* 1937]. At the time that these experiments were carried out it was concluded that the adsorption process itself was responsible for the isomerization of both the α and the β -carotenes, although Zechmeister & Cholnoky [1937], Willstaedt & With [1938] and Euler *et al.* [1938] have been unable to observe isomerization of β -carotene by repeated adsorption alone.

Zechmeister & Tuzson [1938, 1, 2] have recently reported that, like the α - and β -carotenes, lycopene and kryptoxanthin readily isomerize into new carotenoids which they have named neolycopene and neokryptoxanthin respectively. At the same time these workers have also confirmed the isomerization of β -carotene into $\psi\alpha$ -carotene. Thus isomerization is definitely known to occur in at least four members of the carotenoid group, whilst, in addition zeaxanthin undergoes a change that may yet prove to be analogous [Strain, 1938]. It is therefore probable that there exists a complete series of these isocarotenoid pigments.

Zechmeister & Tuzson [1938, 1, 2], in studying the isomerization of lycopene, have made the important new observation that the phenomenon is not brought about by the adsorption process but is actually spontaneous and that the only action of the adsorbent is to separate the isomerides already formed. Thus they have shown that lycopene in solution slowly changes into an equilibrium mixture of lycopene and neolycopene (detected by change in absorption spectrum or by separation on adsorption), but that the change can be brought about much more quickly by heating in benzene for 30 min. These investigators have made an examination of the β -carotene- $\psi\alpha$ -carotene change sufficient to show that, like lycopene, β -carotene isomerizes spontaneously, although more slowly than does lycopene.

In view of the new approach to the problem provided by the work of Zechmeister & Tuzson and the biological importance of β -carotene as a vitamin A precursor as well as its use as an international standard for the measurement of vitamin A activity by biological methods, it seemed of importance to re-examine the isomerization of this substance into $\psi\alpha$ -carotene, particularly since the change appears to be a spontaneous one that might alter the nature of carotene preparations.

In our previous experiments [Gillam & El Ridi, 1936] we prepared $\psi\alpha$ -carotene from β -carotene by adsorption of petrol-benzene solutions of the pigment on alumina. If no separation into two coloured zones occurred the adsorbed pigment was eluted with alcohol-light petroleum, the alcohol removed and the solution *concentrated by evaporation*, then re-adsorbed on alumina. The lower zone contained $\psi\alpha$ -carotene and the upper, β -carotene, which was eluted separately, *concentrated* and re-adsorbed, when good separation into two zones was again obtained.

In planning a test experiment it seemed probable from the work of Zechmeister & Tuzson that the cause of the observed isomerization in our earlier experiments was due to the heating of the solutions during concentration between one adsorption and the next rather than to the adsorption process itself. Accordingly, it was planned to take a definite weight of carotene in light petroleum, pass it through a column of alumina, elute with benzene, concentrate *in vacuo* at not more than 25° , re-adsorb and elute as before. In order to reduce the effect of the time factor in the isomerization it was also planned to adsorb the carotene as many times as possible in one day. The experiment was finally carried out as follows.

2.3 mg. of β -carotene were dissolved in 2.5 ml. of benzene¹ and 2.5 ml. of light petroleum added. The solution was run on to a column of alumina (2.5×28 cm.; one part of active alumina—Merck's "Aluminium-oxyd standardisiert zur chromatographischen Adsorptionsanalyse nach Brockmann"—to two parts of inactive alumina as diluent).

The adsorbed pigment was washed down the column with light petroleum, no separation into two zones being observed. 5 ml. of benzene were then added, followed by light petroleum until the carotene was completely eluted. The solution was concentrated at 25° *in vacuo* and again adsorbed. A very faint separate zone was observed below that of the β -carotene but the percentage of $\psi\alpha$ -carotene was not determinable. The β -carotene fraction was eluted, concentrated and adsorbed a further four times, only slight separation occurring in each case. The subsequent adsorptions gave values for the percentage of $\psi\alpha$ -carotene in the total elutable pigments of 1.5, 1.47, 1.35 and 1.5 respectively (determined spectrophotometrically).

The absorption spectra of the pigments in the upper and lower zones were typical of those previously reported for β -carotene and $\psi\alpha$ -carotene respectively, i.e.

	Absorption maxima (light petroleum B.P. 70–80°)
Upper zone pigment	483 and 452 m μ
Lower zone pigment	477 and 447.5 m μ

(Data obtained on a Hilger-Nutting prism spectrophotometer.)

¹ The benzene serves the double purpose of dissolving the carotene in a smaller volume of solvent as well as of reducing the strength of adsorption of the pigment and so giving greater opportunity of separation of the two components on the column.

It is important to note that when using absorption spectra data to differentiate between carotenoids so similar as the β - and α -carotenes, the locations of the absorption maxima are very sensitive to change of solvent. Thus, if two solutions of β -carotene be made up in light petroleum, B.P. 60–80° and B.P. 40–60°, respectively, the absorption spectra of the two solutions will compare very closely with those of β -carotene and α -carotene (or $\psi\alpha$ -carotene), respectively. This is an obvious source of confusion in work on adsorption of carotenes from light petroleum solution.

The foregoing experiments make it quite clear that repeated adsorption alone is not the cause of the isomerization of β -carotene, a finding that confirms the conclusions of Zechmeister & Tuzson.

In view of the tendency of β -carotene to isomerize spontaneously into $\psi\alpha$ -carotene it is obviously important in any experimental work involving this carotenoid to have precise information regarding the stability of the pigment and the conditions affecting the change into $\psi\alpha$ -carotene. We have accordingly studied the effect of temperature on the isomerization which, from both our own and Zechmeister's observations, is obviously an equilibrium.

Effect of temperature on the change β -carotene to $\psi\alpha$ -carotene

For these experiments recrystallized β -carotene was used (M.P. 181°): intensity of light absorption at 452 m μ in light petroleum (B.P. 70–80°). $E_{1\%}^{1\text{cm}} = 2450$, hence order of purity = 96–98% [cf. Gillam, 1935]. The information most needed was the percentage of $\psi\alpha$ -carotene formed when β -carotene solutions are stored for varying periods of time in the refrigerator, at room temperature, and at the boiling point of the usual solvents (alcohol, light petroleum and benzene). The temperatures finally chosen for study were –2°, 20°, 40°, 60° and 80°.

–2°. Freshly adsorbed β -carotene (i.e. free from $\psi\alpha$ -carotene) was eluted with light petroleum-benzene and immediately cooled to about 0°. The solution was diluted to about 0.05% in equal volumes of light petroleum and benzene and introduced into five brown glass ampoules filled up to the neck and sealed. These were placed in the refrigerator at –2° and one ampoule opened and examined at the end of one, two, four and twelve weeks respectively. After opening each ampoule the solution was allowed to percolate through a column of alumina (active : inactive :: 1 : 1; column 30 × 3 cm.) and washed down with a light petroleum-benzene mixture. The yellow-buff lower zone of $\psi\alpha$ -carotene washed out first and was collected separately from the browner β -carotene zone which followed. The amount of pigment in each of the separate solutions was determined by means of a visual spectrophotometer. A high order of analytical accuracy is not claimed, nor is it to be expected, in these evaluations owing to the easy oxidizability of the pigments, the minute amount of material in use, the method of evaluation and the sometimes slightly imperfect chromatographic separations.

The percentages of $\psi\alpha$ -carotene (on total eluted pigment) were found to be as follows:

1 week	< 1.9 %	4 weeks	< 3.0 %
2 weeks	1.7 %	12 weeks	2.5 %

The differences are not very significant but it is clear that at –2° the isomerization is very slow and that one can store β -carotene in solution for as long as

¹ Unless otherwise indicated the alumina used in all these experiments has been a mixture of one part of active plus two parts of inactive material, this composition having been found by experiment to give the best separations.

three months and be certain that less than 3–4 % of the pigment will be converted into the isomeric form.

Room temperature (ca. 20°). Pure β -carotene crystals were dissolved in light petroleum containing 30 % of benzene and adsorbed immediately. The β -carotene layer was eluted separately and the solution sealed up in brown glass ampoules filled up to the neck and kept in the dark at about 20°. After standing for varying lengths of time the percentage of $\psi\alpha$ -carotene was determined as before, the values obtained being as follows:

1 day	< 1 %	35 days	10.0 %
7 days	5.5 %	49 days	11.1 %
18 days	6.2 %		

Thus carotene solutions kept at room temperature definitely isomerize slowly if only to a few per cent. Even after standing for 7 weeks some 90 % of the pigment is, however, unchanged β -carotene (in the absence of oxygen).

40°. Solutions of β -carotene kept at this temperature gave the following values for $\psi\alpha$ -carotene content: 1 hr. 4 %; 3 hr. 5.4 %; 24 hr. 11.2 %.

60°. In 1 hr. at this temperature 7–8 % of the carotene was isomerized whilst after 3 hr. duplicate experiments showed the presence of 9.1 and 10.3 % respectively of the $\psi\alpha$ -form.

80°. At the boiling-point of light petroleum containing about 50 % of benzene (ca. 77–80°), the amount of $\psi\alpha$ -carotene formed in 1 hr. was found to be 8.0 and 9.0 % respectively, in duplicate experiments. In 3 hr. the values obtained in different experiments were 31.9, 34.1 and 27.0 % respectively, showing that at this temperature the change is relatively rapid. After concentrating solutions of β -carotene for various lengths of time at 77° values of 28.1, 32.0, 29.3 and 33.0 % were obtained in four different cases. These results give a mean value of 30.8 % and as longer boiling produces no higher values this must represent the equilibrium percentage of $\psi\alpha$ -carotene in the reversible isomerization (for these particular conditions).

As calcium hydroxide is at least as effective as alumina for the separation of the two isomerides [cf. Zechmeister & Tuzson, 1938, 2] β -carotene solutions isomerized at 80° were also adsorbed on this adsorbent. Typical values obtained were 28.5 and 29.6 % of $\psi\alpha$ -carotene, i.e. similar to those obtained with alumina.

The reverse change, $\psi\alpha$ -carotene to β -carotene

On the assumption that the spontaneous isomerization of β -carotene into $\psi\alpha$ -carotene is reversible and that the ultimate result achieved is an equilibrium between the two opposing reactions it was decided to check some of the previous values for the percentage of $\psi\alpha$ -carotene produced under given conditions of time and temperature by starting from $\psi\alpha$ -carotene instead of β -carotene.

The solution of pigment was prepared by isomerization of β -carotene by boiling the petrol-benzene solution for some 30 min. and then separating the $\psi\alpha$ -carotene by adsorption as previously described [Gillam & El Ridi, 1936] immediately before the following experiments.

One sample was stored at -2° for two months (under nitrogen) and was then adsorbed on alumina (active : inactive :: 1 : 2). Two duplicate experiments gave 95.0 and 94.0 for the percentages of $\psi\alpha$ -carotene in the mixture. The order of change (5.5 %) is thus comparable with the percentage of β -carotene isomerized

under the same conditions, i.e. 2% (*vide supra*) and the difference in the figures is consistent with the expectation that the change $\psi\alpha$ -carotene to β -carotene would be 2-3 times as fast as the reverse reaction.

After standing for 24 hr. at room temperature (*ca.* 20°) the $\psi\alpha$ -carotene solution was still found to contain 95% of this pigment, but after 2 weeks the value dropped to approximately 80%. After 2 months the $\psi\alpha$ -carotene content was found to be close to 53% thus showing that equilibrium had not yet been reached. (The ultimate value should be 30-31% of the $\psi\alpha$ -form.)

When heated in light petroleum-benzene mixture at the boiling point (*ca.* 80°) the equilibrium state was reached much more quickly from this side of the reaction than from the β -carotene side. Thus after half an hour's heating values of 30.0 and 32.5%, respectively, were obtained for the content of $\psi\alpha$ -carotene and further heating did not alter these values significantly. The mean value, 31.3%, compares reasonably well with the percentage of 30.8 found for the equilibrium value when starting from the β -carotene side of the reaction.

Separation of β -carotene and $\psi\alpha$ -carotene

Since it has been pointed out by Zechmeister & Tuzson that the conversion of β -carotene into $\psi\alpha$ -carotene is spontaneous and accelerated by heat, it follows that the best method of preparation of the latter pigment is to heat β -carotene in light petroleum or benzene for about an hour at the boiling point when the equilibrium mixture containing some 30% of the $\psi\alpha$ -form is obtained. Since it is also clear that adsorption alone does not bring about the change simple chromatography can be used to separate the two forms [cf. Gillam & El Ridi, 1936]. On the other hand crystallization of the equilibrium mixture from light petroleum yields solid β -carotene leaving the mother liquor temporarily richer in $\psi\alpha$ -carotene. On standing, or on heating, the $\psi\alpha$ -carotene percentage drops to that of the equilibrium state and is ready to deposit more β -carotene. The following experimental data illustrate these points.

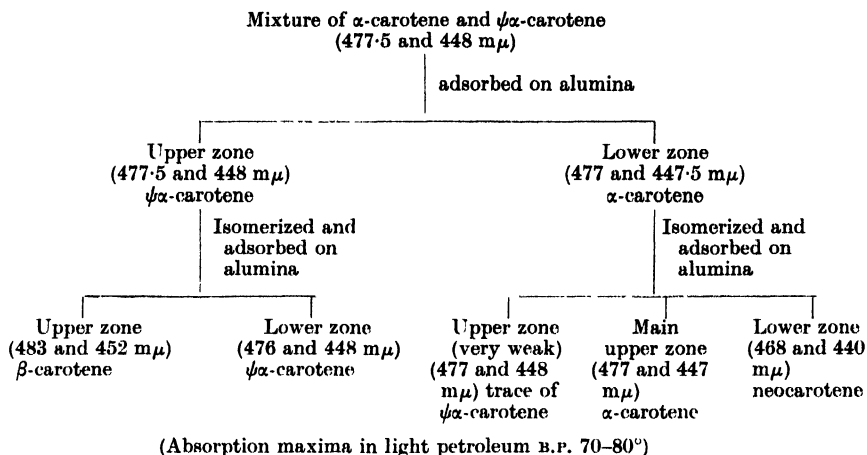
(a) 30 mg. β -carotene in 75 ml. light petroleum were heated at 70-80° for 2 hr. On evaporation to crystallization-point 5 mg. β -carotene (containing < 1% $\psi\alpha$ -carotene) separated. The mother-liquor contained 40% of $\psi\alpha$ -carotene but on heating at 80° for 1 hr. this value dropped to 30.2%.

(b) 27 mg. β -carotene (determined spectrophotometrically and free from $\psi\alpha$ -carotene) were partially isomerized by heating in light petroleum. ($\psi\alpha$ -carotene determined by adsorption = 26.0%.) The solution on crystallization yielded 5.5 mg. of β -carotene. The mother liquor contained 21.5 mg. of "carotene" of which 33.8% was found to be present as the $\psi\alpha$ -isomeride.

Separation and differentiation of α -carotene and $\psi\alpha$ -carotene

In the crystalline condition these two pigments are distinguishable by differences in melting point and rotation [cf. Gillam & El Ridi, 1936; Gillam *et al.* 1937]. In solution they are more difficult to differentiate, particularly as they exhibit absorption maxima at the same wave-lengths. On adsorption on alumina they separate fairly easily, the α -carotene being less strongly adsorbed [cf. Karrer *et al.* 1937]. In mixtures of β -, α - and $\psi\alpha$ -carotenes the $\psi\alpha$ -carotene is adsorbed, between the β - and α -isomerides. The two α -forms can be differentiated in solution by the fact that, on heating, α -carotene gives a mixture of α - and neo-carotenes whilst $\psi\alpha$ -carotene gives a mixture of $\psi\alpha$ - and β -carotenes, the two pairs being easily differentiated after adsorption by the location of the absorption maxima

of the eluted pigments. The following experimental data are typical and provide the support for these statements.



It is of interest to enquire whether the isomerization of β -carotene into $\psi\alpha$ -carotene occurs in the solid state or only in solution. All the evidence goes to show that the change occurs only in solution; thus, samples of solid β -carotene kept in a refrigerator for periods of from 3 months to 2 years have yielded on solution less than 1% of $\psi\alpha$ -carotene when the solutions were examined immediately after preparation. The spontaneous change must therefore only commence immediately after the crystals are dissolved.

Further investigations are proceeding on the isomerization of α -carotene into neocarotene and neo- α -carotene.

SUMMARY

Earlier experiments on the effect of chromatographic adsorption on the α - and β -carotenes showed that these pigments readily undergo isomerization into new carotenoids. Thus, β -carotene gives $\psi\alpha$ -carotene, whilst α -carotene yields neocarotene and neo- α -carotene. It was originally thought that the actual adsorption process brought about the isomerization, but recent experiments by Zechmeister & Tuzson have shown that lycopene and kryptoxanthin can also change into new pigments and, moreover, that the change is spontaneous and not brought about by adsorption.

In the present investigation the isomerization of β -carotene has been re-examined in the light of these new observations. The suggestion of Zechmeister & Tuzson that the isomerization of β -carotene is also spontaneous and not due to adsorption, has been confirmed as also has the fact that the change is an equilibrium between two opposing reactions. The effect of temperature on the forward reaction (β -carotene to $\psi\alpha$ -carotene) has been studied and it has been found that whereas at -2° some 2.5% of the carotene is isomerized in 12 weeks, at room temperature some 11% is changed in only 7 weeks. At 80° the isomerization reaches equilibrium in less than 3 hr. when some 31% of $\psi\alpha$ -carotene is present.

The reverse change of $\psi\alpha$ -carotene to β -carotene has been found to proceed more rapidly. Even so, only 5–6% of this pigment, in solution, is changed in 2 months at -2° . At 80° the equilibrium is reached in about $\frac{1}{2}$ hr.

In order to reduce the tendency of carotene solutions to isomerize spontaneously they should therefore be stored as near to 0° as possible and concentration of the solutions should only be carried out at low temperature. When partial isomerization has occurred one adsorption on alumina or calcium hydroxide immediately before use gives β -carotene in pure form, whilst crystallization of the equilibrium mixture yields practically pure β -carotene in each crop of crystals, provided that the mother liquor is brought to equilibrium by raising to the boiling point between crystallizations.

α -Carotene and $\psi\alpha$ -carotene can be separated and differentiated by chromatographic methods with absorption spectra control.

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CLXII. ESTIMATION OF LACTIC ACID IN BIOLOGICAL MATERIAL BY OXIDATION WITH CERIC SULPHATE

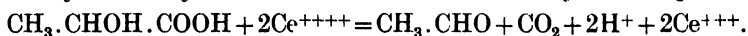
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(Received 30 June 1939)

FROMAGEOT & DESNUELLE [1935] proposed a method of estimation of pyruvic acid based on the oxidation of α -ketonic acids by ceric sulphate. The value of the method is diminished by the fact that numerous substances commonly encountered in biological material, e.g. lactic and citric acids, also react with ceric sulphate. The interference by lactic acid is not serious so long as the amount of the latter is small compared with that of the pyruvic acid present and so long as the temperature at which the reaction is carried out is kept low (e.g. approx. 0°). Citric acid is attacked by ceric sulphate even at low temperatures and its possible presence must be taken into account in any estimation of pyruvic acid by ceric sulphate oxidation.

Whilst investigating the magnitude of the error in pyruvic acid estimations caused by the presence of lactic acid in tissue extracts, we found that at relatively high temperatures, e.g. 50°, lactic acid is oxidized quantitatively by ceric sulphate to acetaldehyde. Analysis showed that the reaction obeyed the equation



Lactic acid could be estimated with accuracy by allowing it to react with an excess of standard ceric sulphate solution at 50° for a suitable time and titrating the excess Ce^{++++} with standard ferrous ammonium sulphate solution. This procedure is of little value when applied to biological material owing to the large errors introduced by the presence of other substances attacked by ceric sulphate under the conditions employed. Estimation, however, of the acetaldehyde produced by the oxidation of lactic acid showed that its amount was not materially affected by the presence, in the lactic acid solution, of substances commonly encountered in tissue extracts, so long as excess ceric sulphate was used.

The method we have adopted for the estimation of lactic acid consists of the oxidation of the substance by ceric sulphate to acetaldehyde, followed by estimation of the latter by absorption in sodium bisulphite solution as in the well-known method of Friedemann *et al.* [1927]. We find that the presence of glucose, fructose, starch and a variety of other substances does not interfere with the lactic acid estimation so long as these are not present in abnormally large concentrations. Hence it is unnecessary to remove these substances from tissue extracts before making lactic acid estimations. An estimation of lactic acid in blood requires only treatment with trichloroacetic acid, an aliquot of the filtrate being treated at once with ceric sulphate solution. The same procedure is carried out with tissue brei, e.g. liver or brain.

Details of the estimation of lactic acid by ceric sulphate oxidation

We have found it convenient to use for this estimation the well-known Schrödter flask (Fig. 1), which, besides being relatively inexpensive, takes up little space and can be easily cleaned. Moreover, a battery of such flasks can be

used at once so that a large number of estimations can be carried out quickly. The end of the dropping funnel (A, Fig. 1) is bent so that it nearly touches the bottom of the flask.

5 ml. of the fluid, of which the content of lactic acid is to be estimated, are placed in the main vessel (C) of the apparatus. In the dropping funnel (A) are placed 5 ml. 10% ceric sulphate¹ in N H_2SO_4 solution. 5 ml. of 1% sodium bisulphite solution are placed in the absorption tube (B) of the apparatus. The apparatus is placed in a water-bath, or thermostat, kept at 50°. It is so arranged that the absorption tube (B) is well above the water level in the thermostat, the temperature of the contents of the tube being kept as near that of the room as possible. The dropping funnel (A) is now connected to a nitrogen cylinder, the tap of the funnel opened and a slow stream of nitrogen (3 or 4 bubbles a second) is passed through the apparatus. We have found that 60–90 min. are ample for the completion of the reaction and for the complete sweeping out of the acetaldehyde from the reaction vessel into the bisulphite solution, using the quantities of lactic acid mentioned later in this paper. If nitrogen is passed through the flask too quickly, there is incomplete absorption of acetaldehyde by the bisulphite.

If it is found that the ceric sulphate in the reaction flask becomes nearly, or completely, reduced during the course of the experiment, it will be necessary to commence the experiment again using either less of the fluid containing the lactic acid, or a more concentrated solution of ceric sulphate. It is essential that at the termination of the experiment, there should still be excess of ceric sulphate present in the reaction flask.

At the end of the allotted time, the contents of the absorption tube are washed into a beaker. This is easily performed when making use of the type of Schrödter flask in which the reaction vessel is connected with the rest of the apparatus with a ground glass joint (as in Fig. 1). The free bisulphite is titrated with $N/10$ iodine solution and the bound bisulphite, after treatment with $NaHCO_3$, with $N/50$ iodine solution, the procedure being precisely the same as that in the method of Friedemann *et al.* The apparatus used in the latter method can obviously be used in place of the one described above.

Accuracy of the method. The error of the method appears not to be greater than $\pm 5\%$ when using quantities of lactic acid varying from 0.15 to 1.50 ml. of 0.032 M solution (i.e. 0.4–4 mg. lactic acid). Typical results are shown in Table I.

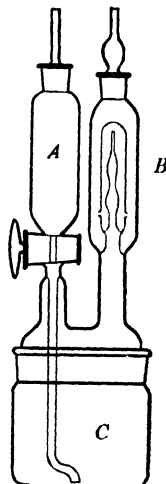


Fig. 1.

Table I

Vol. of 0.032 M sodium lactate solution taken ml.	$N/50$ iodine required observed ml.	$N/50$ iodine required calculated ml.	% error
1.5	4.72	4.8	-1.7
1.0	3.14	3.2	-1.9
1.0	3.06	3.2	-4.4
1.0	3.15	3.2	-1.6
0.5	1.64	1.6	+2.5
0.25	0.83	0.8	+3.8
0.15	0.50	0.48	+4.0

¹ We have found the technical grade of ceric sulphate (B.D.H.) to be quite satisfactory. The solution should be filtered before use.

Effects of impurities. The effects of the addition of a variety of substances to a zinc lactate solution on the estimation of the lactic acid by ceric sulphate oxidation were investigated and the results are shown in Table II. The substances included glucose, fructose, starch, pyruvic acid, malic acid, citric acid, ethyl alcohol, oxalic acid, β -hydroxybutyric acid, alanine, malonic acid and tartronic acid, and were added in quantities which might be expected in investigations on biological material. The error of the method did not exceed $\pm 5\%$. The estimation can be carried out with accuracy in presence of trichloroacetic acid. The presence of urea or of nutrient peptone broth as used in bacteriological work introduced no error.

Table II

Solutions consisted of mixtures of 1 ml. 0.39% anhydrous zinc lactate solution and the volumes of solutions mentioned below, the total volume being made up to 5 ml. with water. The theoretical iodine titration, corresponding to the lactate taken was 3.20 ml. *N*/50.

Solution added to the lactate	<i>N</i> /50 iodine titration observed	% error
Nil	3.20	0.0
1 ml. 0.2% <i>dl</i> -alanine	3.20	0.0
1 ml. 20% trichloroacetic acid	3.06	-4.3
1 ml. 0.2% glucose	3.06	-4.3
1 ml. 0.2% fructose	3.04	-4.9
0.5 ml. 1% starch	3.25	+1.6
2 ml. 0.3% ethyl alcohol	3.14	-1.9
2 ml. 0.2% pyruvic acid	3.35	+4.7
1 ml. 0.2% malic acid	3.10	-3.3
1 ml. 0.1% citric acid	3.22	+0.5
2 ml. 0.2% tartronic acid	3.12	-2.5
1 ml. 0.2% malonic acid	3.17	-1.0
1 ml. 0.2% oxalic acid	3.04	-4.9
2 ml. 0.2% β -hydroxybutyric acid	3.08	-3.7
0.1 g. urea	3.10	-3.3
1 ml. nutrient peptone broth	3.26	+1.9

The presence of large concentrations of some of these substances, e.g. citric acid, does introduce errors, these being largely due to the reduction of the concentration of the ceric sulphate. If such large concentrations of impurities are expected to be present, the initial concentration of ceric sulphate may be increased to 20%, or the solutions under investigation may be diluted.

Estimations of lactic acid in blood

Human blood, treated with oxalate (20–30 mg. potassium oxalate per 10 ml. blood), is treated with an equal, or approximately equal, volume of 20% trichloroacetic acid. The mixture is centrifuged, and lactic acid estimated directly on an aliquot of the centrifugate. Convenient quantities are 5 ml. blood, and 5 ml. 20% trichloroacetic acid, 5 ml. of the centrifugate being taken for the estimation. Some typical results are shown in Table III. The amount of oxalate used to prevent clotting does not interfere with the estimation. Citrate should not be used to prevent clotting as the presence of this in relatively large quantity causes too great a reduction of the ceric sulphate.

Lactic acid added to blood can be estimated with fair accuracy as shown in Table III and it is evident that the presence of glucose etc. in the blood has not caused any serious error.

Table III. *Lactate estimations on whole blood*

Exp.	
1 a	5 ml. freshly drawn oxalated human blood, 1 ml. water and 5 ml. 20% trichloroacetic acid; mixture centrifuged, and lactate estimated on 5 ml. centrifugate. $N/50$ iodine required = 0.34 ml. This is equivalent to $0.34 \times \frac{11}{5} \times \frac{100}{5} \times 0.9 = 13.4$ mg. lactic acid in 100 ml. whole blood.
1 b	5 ml. of the blood used in exp. 1 a, 1 ml. (anhydrous) 0.39% zinc lactate (= 2.88 mg. lactic acid) and 5 ml. 20% trichloroacetic acid; lactate estimated on 5 ml. centrifugate. $N/50$ iodine required = 1.70 ml. Therefore $\frac{11}{5} (1.70 - 0.34) = 2.99$ ml. $N/50$ iodine = titre of iodine equivalent to lactate added to the blood. This is equivalent to 2.69 mg. lactic acid representing 94% of the added lactic acid.
2 a	5.0 ml. oxalated human blood, added 1 ml. water and 5.0 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. $N/50$ iodine required = 0.35 ml. This is equivalent to $0.35 \times \frac{11}{5} \times \frac{100}{5} \times 0.9 = 13.8$ mg. lactic acid in 100 ml. whole blood.
2 b	5.0 ml. oxalated human blood used in exp. 2 a, 1 ml. 0.39% zinc lactate solution and 5.0 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. $N/50$ iodine required = 1.72 ml. Therefore $\frac{11}{5} (1.72 - 0.35) = 3.01$ ml. $N/50$ iodine; theoretical titre = 3.20 ml.
3 a	2.4 ml. whipped, freshly obtained, rat blood and 5 ml. 20% trichloroacetic acid; mixture filtered, and precipitate washed thoroughly with water; lactate estimated on the combined filtrate and washings. $N/50$ iodine required = 0.77 ml. Therefore lactic acid present in whole rat blood = $0.77 \times \frac{100}{2.4} \times 0.9 = 28.8$ mg./100 ml.
3 b	2 ml. whipped, fresh, rat blood, 1 ml. 0.39% zinc lactate solution and 5 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 3 ml. centrifugate. $N/50$ iodine required = 1.45 ml. Therefore lactic acid present in the mixture before centrifuging = $1.45 \times \frac{8}{3} \times 0.9$ mg. = 3.48 mg. From exp. 3 a lactic acid present in the blood = $\frac{28.8}{50} = 0.58$ mg. Therefore added lactic acid estimated = $3.48 - 0.58 = 2.90$ mg. The amount actually added was 2.88 mg.

Estimation of lactic acid in cerebrospinal fluid

Estimations of lactic acid in cerebrospinal fluid were made without any preliminary treatment with trichloroacetic acid. 5 ml. of the fluid were placed in the reaction vessel and treated at once with the ceric sulphate reagent in the manner already described. Results are shown in Table IV. Lactic acid added to the cerebrospinal fluid was estimated with an error of $\pm 5\%$.

Table IV

Exp.	Solution in reaction flask	$N/50$ iodine titration observed ml.	Lactic acid content mg./100 ml.
1	5 ml. cerebrospinal fluid of case A	1.03	18.5
2	5 ml. cerebrospinal fluid of case A + 0.5 ml. zinc lactate (0.39%) solution	2.72 (theoretical = 2.63 ml.)	
3	2 ml. cerebrospinal fluid of case B	0.37	16.6
4	2 ml. cerebrospinal fluid of case B + 0.5 ml. zinc lactate (0.39%) solution	1.90 (theoretical = 1.97 ml.)	

Estimations of lactic acid in tissues

Minced liver or brain was suspended in phosphate buffer solution or saline and treated at once with trichloroacetic acid. The mixture was centrifuged and lactic acid estimated on an aliquot of the filtrate. No special precautions were taken in these experiments to prevent the breakdown of tissue glucose or glycogen into lactic acid. Lactic acid added to the tissue brei was estimated with reasonable accuracy. Details and typical results of experiments are shown in Table V.

Table V. *Lactate estimations on tissues*

Exp.	
1 a	<p>4 g. minced rat liver suspended in <i>M/5</i> phosphate buffer pH 7.4 to make a total vol. of 12 ml. 5.5 ml. treated with 1 ml. water and 4.5 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. <i>N/50</i> iodine required = 0.32 ml.</p> <p>This is equivalent to $0.32 \times \frac{11}{5} \times \frac{12}{5.5} \times \frac{100}{4} \times 0.9 = 34.5$ mg. lactic acid/100 g. rat liver tissue.</p>
1 b	<p>5.5 ml. liver suspension prepared in exp. 1 a, 1 ml. 0.39% zinc lactate and 4.5 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. <i>N/50</i> iodine required = 1.71 ml.</p> <p>Therefore lactic acid present in mixture before centrifuging = $1.71 \times \frac{11}{5} \times 0.9 = 3.38$ mg.</p> <p>Lactic acid present in the liver suspension = $0.32 \times \frac{11}{5} \times 0.9$ (from exp. 1 a) = 0.63 mg.</p> <p>Therefore added lactic acid estimated = $3.38 - 0.63 = 2.75$ mg. The amount of lactic acid actually added was 2.88 mg.</p>
2 a	<p>5 g. minced sheep brain cortex suspended in <i>M/5</i> phosphate buffer pH 7.4 to make a total vol. of 15 ml.; 5 ml. of the suspension, 1 ml. water and 5 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. <i>N/50</i> iodine required = 0.76 ml.</p> <p>This is equivalent to $0.76 \times \frac{11}{5} \times \frac{15}{5} \times \frac{100}{5} \times 0.9 = 90.3$ mg. lactic acid/100 g. sheep cortex tissue.</p>
2 b	<p>5 ml. suspension prepared in exp. 2 a, 1 ml. 0.39% zinc lactate and 5 ml. 20% trichloroacetic acid; mixture centrifuged and lactate estimated on 5 ml. centrifugate. <i>N/50</i> iodine required = 2.22 ml.</p> <p>Therefore lactic acid present in mixture before centrifuging = $2.22 \times \frac{11}{5} \times 0.9 = 4.40$ mg.</p> <p>Lactic acid present in brain suspension = $0.76 \times \frac{11}{5} \times 0.9$ (from exp. 2 a) = 1.50 mg.</p> <p>Therefore added lactic acid estimated = $4.40 - 1.50 = 2.90$ mg. The amount of lactic acid actually added was 2.88 mg.</p>

Estimation of lactic acid in urine

It is advisable when making estimations of lactic acid in urine to use as oxidant 20% ceric sulphate in $N H_2SO_4$ solution. Insufficient work has been carried out to determine the order of accuracy of lactic acid estimations in urine by ceric sulphate oxidation, but a few experiments have shown that relatively small quantities of added lactic acid can be estimated with fair accuracy. Typical results are shown in Table VI. In these experiments the urine is treated at once with the ceric sulphate reagent without any preliminary treatment other than filtration.

Table VI. *Lactate estimation in urine*

Exp.	
1	Lactic acid estimated directly on 5 ml. filtered urine. N/50 iodine required = 0.35 ml. This is equivalent to $0.35 \times \frac{100}{5} \times 0.9 = 6.3$ mg./100 ml.
2	5 ml. filtered urine, added 0.5 ml. 0.39% zinc lactate; lactic acid estimated directly. N/50 iodine required = 1.85 ml. This is equivalent to $1.85 \times 0.9 = 1.67$ mg. lactic acid. Lactic acid present in 5 ml. urine (from exp. 1) = 0.31 mg. Therefore added lactic acid estimated = $1.67 - 0.31 = 1.36$ mg. The amount of lactic acid added was 1.44 mg.

Lactic acid estimation in presence of ferricyanide

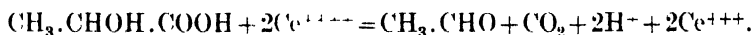
The presence of ferricyanide introduces a large error into a lactic acid estimation when using acid ceric sulphate as oxidant; ferricyanide may be easily removed by adding to the solution, made acid with trichloroacetic acid, ferrous ammonium sulphate solution, and a drop of ferric chloride solution if the presence of ferrocyanide is suspected. The amount of ferrous ammonium sulphate added should be just sufficient to react with the ferricyanide present.

The mixture is well shaken, centrifuged and lactic acid estimated on an aliquot of the centrifugate.

Lactic acid added to a 2% solution of potassium ferricyanide was estimated in this way with 94% accuracy.

SUMMARY

1. Lactic acid is oxidized by ceric sulphate in acid solution according to the equation



2. The reaction proceeds rapidly to completion at 50° when using the quantities and concentrations of lactic acid described in this paper.

3. The reaction is made the basis of a method for lactic acid estimation in biological material, the acetaldehyde produced being absorbed by sodium bisulphite and estimated iodimetrically.

4. The formation of acetaldehyde is independent of the presence of a variety of substances commonly encountered in biological material, e.g. glucose, malic acid etc., and no preliminary treatment of tissue fluids other than precipitation of protein by trichloroacetic acid is required for the estimation of lactic acid.

5. Details of the method as applied to the estimation of lactic acid in blood, cerebrospinal fluid, urine and tissue brei are described. The error in the estimation of lactic acid does not exceed $\pm 5\%$.

Our thanks are due to the Medical Research Council for an assistant grant to one of us (J. J. G.) and for a grant in aid of the equipment of this laboratory.

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CLXIII. AMINE OXIDASE

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In a previous paper it was shown that animal tissues contain an enzyme, amine oxidase, which oxidizes a number of aliphatic and aromatic amines, including adrenaline [Blaschko *et al.* 1937]. This enzyme is distinct from the diamine oxidase, or histaminase, which oxidizes diamines [Zeller, 1938].

Amine oxidase was shown to occur in mammalian liver, intestine, kidney, brain and lung. A more detailed study has now been made of the distribution of the enzyme in mammalian tissues. In addition the previous observations on the specificity of the enzyme have been extended by testing a number of new amines as substrates.

I. Distribution of amine oxidase in mammalian tissues

Tissue extracts were prepared as described by Blaschko *et al.* [1937] by grinding with sand, centrifuging, dialysing and making up to 3 vols. with phosphate buffer of pH 7.3. The experiments were carried out with Warburg-Barcroft manometers: each vessel contained 1.9 ml. tissue extract, 0.1 ml. *M*/50 HCN and 0.2 ml. *M*/4 amine hydrochloride solution.

Table I. *Amine oxidase content of mammalian tissues*

Oxygen uptake (μ l. O₂ 0.63 g. fresh tissue/hr. at pH 7.3 and 37°).

Animal	Organ	Tissue extract alone	Increased uptake with			
			Tyramine	<i>iso</i> Amylamine		
Pig	Liver	0	162	158		
	Pancreas	2	47	—		
	Heart	7	21	28		
	Intestine	5	59	—		
	Spleen	18	9	9		
	Thyroid	2	4	—		
	Kidney	37	285	—		
Ox	Liver	8	501	315		
	Heart	3	33	28		
	Intestine	23	175	69		
	Spleen	14	61	—		
	Kidney	55	412	258		
	Brain	13	16	31		
Sheep	Liver	5	387	289		
	Pancreas	2	11	—		
	Heart	3	4	—		
	Intestine	2	104	—		
	Spleen	2	57	33		
	Thyroid	10	27	—		
	Kidney	7	349	266		
	Brain	12	55	40		
Guinea-pig	Testicle	Tissue alone	With tyramine	With <i>iso</i> amyl- amine	With hordenine	With <i>p</i> -sympatol
		0	24	17	1	8

The results of these experiments are given in Table I. In all the animals examined, liver, kidney and intestine show the highest activity; but most other organs examined were active to some extent. The relative activities of different organs in different species differ widely: for instance, the pig's heart is much more active than the sheep's heart, where the extra O_2 uptake in the presence of amines was found to be only just outside the limits of experimental error. In another experiment (not included in Table I) no enzymic activity was found in a preparation from dog's ventricle.

A number of similar experiments are not included in Table I as they were done under slightly different conditions. Among these are observations on the uterus, which contains considerable quantities of the enzyme.

One part of dog's uterus was ground with sand, 1 part phosphate buffer was added; the mixture was centrifuged for 5 min., the supernatant fluid being used for the experiment. 1.7 ml. of this preparation were incubated with 0.1 ml. $M/50$ HCN and 0.2 ml. $M/4$ amine hydrochloride. The O_2 uptakes in 45 min. were:

	μ l.
Extract alone	7
Excess with tyramine	51
Excess with <i>isoamylamine</i>	38
Excess with <i>l-p-sympatol</i>	19

The activity of dog uterus, when compared with the other data given, is quite considerable. Sheep uterus was also tested; it contains appreciable amounts of the enzyme.

The adrenals also contain amine oxidase. In an experiment, in which to 1 part of sheep adrenals 1 part of phosphate buffer was added, 1.7 ml. of the extract (with 0.1 ml. $M/50$ HCN and 0.2 ml. $M/4$ amine hydrochloride) the subsequent O_2 uptakes in 55 min. were:

	μ l.
Extract alone	106
Excess with tyramine	88
Excess with <i>isoamylamine</i>	43
Excess with <i>l-p-sympatol</i>	17

The relatively large O_2 uptake of the extract alone makes these figures somewhat less conclusive.

Guinea-pig skeletal muscle contains only small amounts of enzyme. 1.7 ml. of a preparation from guinea-pig muscle (1 part muscle + 2 parts phosphate buffer) showed in the presence of 0.1 ml. $M/50$ HCN and 0.2 ml. $M/4$ amine hydrochloride the following O_2 uptakes/hr.:

	μ l.
Extract alone	14
Excess with tyramine	8
Excess with <i>isoamylamine</i>	7
Excess with tryptamine	12

The experiments reported in this and the preceding paper show that the enzyme has a much wider distribution than was previously believed. Kidney, liver and intestine contain the enzyme in highest concentration, but lungs, brain, uterus and—in some animals—spleen also give highly active preparations. The extent to which cortex and medulla of the adrenal contribute to the total activity of the organ remains to be examined. In view of the wide distribution the lack of enzymic activity of muscular tissue, especially skeletal muscle, is interesting. But here again species differences in the distribution of the enzyme may exist. The uterus is the only muscular organ that seems regularly to

contain amine oxidase in high concentration; this is of interest in view of the observation of Ewins & Laidlaw [1910] that tyramine disappears from the perfused cat's uterus. These authors found that tyramine is metabolized to *p*-hydroxyphenylacetic acid, which indicates that the disappearance of the amine is due to the amine oxidase.

How do the observations on the distribution of the enzyme contribute to the understanding of its function in the animal body? It has already been pointed out that the presence of the enzyme in the intestine may serve to protect the body from the effects of amines formed by bacterial activity in the intestinal lumen. This is supported by the fact that the diamine oxidase also occurs in high concentration in the intestine and may therefore serve to prevent the diamines (histamine, putrescine, cadaverine) from reaching the general circulation. It appears unlikely that an enzyme system which shows such a widespread distribution should have only a localized detoxicating function, and the amine oxidase may well have a more general significance in cell metabolism.

Another function has been suggested for this enzyme in the work of Gaddum & Kwiatkowski [1938], namely that it may serve to inactivate the adrenergic transmitter substance of the post-ganglionic sympathetic neurones released on stimulation of the sympathetic nerve. This theory will be dealt with more fully elsewhere, but it must be said that the experiments reported here do not support the conception. A number of organs with a good sympathetic nerve supply, it is true, are rich in amine oxidase (e.g. the intestine, liver and uterus); but on the other hand heart muscle is poor in enzyme and the brain contains relatively large amounts. The sympathetic supply to the rabbit's ear on which Gaddum & Kwiatkowski's experiments were done, is probably mostly to the blood vessels of the skin; attempts to demonstrate the presence of the enzyme in the skin from rabbit's ears failed, although it must be admitted, in view of the toughness of the tissue, that it is possible that the enzyme may not have been successfully extracted.

II. *Specificity of amine oxidase*

Blaschko *et al.* studied the specificity of the guinea-pig oxidase by testing the oxidation of 66 amines of different types. A number of new amines have now been tested. The conditions in these experiments were as previously described. The results shown in Table II agree with and confirm the conclusions previously

Table II

Substrate	Tissue tested (l = liver, i = intestine)	Relative rate
67. <i>l</i> -sec-Butylamine	l, i	<2
68. Novocain	l, i	<2
69. Glucosamine	l, i	<2
70. Camphylamine	l, i	3
71. Piperidine	l, i	<2
72. 3:4-Methylenedioxy- δ -phenyl- β -aminobutane	l, i	<2
73. α -Phenylethylamine	l, i	<2
74. Hordenine methylchloride	l, i	<2
75. β -Phenylethylmethylamine	l	29
76. β -(3-Methoxyphenyl)-ethylamine	l	24
77. β -(3:4-Dimethoxyphenyl)-ethylamine	l	9
78. Ethoxy-6-methylaminomethyl-2-cumarane (887 F)	l	<2
79. Methylaminomethyl-2-cumarane (879 F)	l	<2
80. Piperidinomethyl-3-benzodioxane (933 F)	l	<2
81. ϵ -Aminocaproic acid	l	<2
82. Agmatine	l	<2

arrived at as to the specificity of the amine oxidase. The absence of any oxidation with *l*-sec-butylamine shows that the inability of the enzyme to oxidize compounds of the type $R(\text{CH}_3)\text{CHNH}_2$ extends to the aliphatic as well as to the aromatic series of amines.

This result had previously been established only for aromatic compounds such as ephedrine. A number of ephedrine derivatives which have since been tested all conform to this general rule. A further point of interest is in the behaviour of the methoxy-phenyl derivatives: the monomethoxy compound No. 76 shows a relative rate 24, the dimethoxy compound, No. 77 gives 9, and the trimethoxy compound mescaline (No. 58 of our preceding paper) has a relative rate of 5. This shows that the relative rate decreases with increasing substitution by methoxyl groups.

III. *Effect of urea on the enzyme*

That urea has a denaturing action on certain proteins is well known. Guinea-pig liver preparations are inactivated by treatment with concentrated urea solutions.

Equal amounts of guinea-pig liver extract and 6*M* urea solution were mixed and the enzymic activity towards tyramine was tested immediately. The activity of the extract+urea was found to be only 24% of that of the extract alone. Another sample of the mixture was tested after keeping in the refrigerator for 24 hr.: it showed no activity. The remainder of the extract-urea mixture was then dialysed against distilled water for 24 hr. to remove the urea; no activity had reappeared when tested. This makes it likely that urea irreversibly inactivates the enzyme. In this respect the amine oxidase differs from succinic dehydrogenase, which has recently been shown to retain its activity after treatment with concentrated urea solutions [Hopkins *et al.* 1939].

SUMMARY

1. The enzyme amine oxidase is shown to have a very wide distribution in the mammalian body.
2. The list of substances tested as possible substrates has been extended; the results confirm the conclusions previously arrived at.
3. Urea (3*M*) irreversibly inactivates the enzyme.

The authors wish to thank Sir F. G. Hopkins, Sir J. Barcroft and Prof. Golla for their interest, and Dr D. Bovet, Prof. J. A. Gunn and Dr Kipping for valuable gifts of chemicals. One of us (K. B.) thanks the University of Bombay for Springer Research and Sir Nathubhai Scholarships, one of us (H. B.) thanks the Ella Sachs Plotz Foundation, and one of us (D. R.) the Rockefeller Foundation, for grants.

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CLXIV. THE MOLECULAR WEIGHT OF CRYSTALLINE MYOGEN¹

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(Received 17 June 1939)

BARANOWSKI [1939] has recently obtained from rabbit muscle extract a crystalline protein, which he has named myogen A. As this is one of the few tissue proteins ever crystallized, it was of particular interest to investigate its molecular weight and its homogeneity by ultracentrifugal methods [Svedberg, 1939].

Experimental. The sedimentation constant of crystalline myogen was determined in the ultracentrifuge at 69,000 r.p.m., corresponding to a force of about 350,000 times gravity. The ordinary scale method of Lamm [1937] was used for the observations. For refractometric determinations of the protein concentration during the centrifuge runs it was necessary to know the refractive index increments dn/dc of the myogen solutions. These were measured in a differential double prism with pure water as reference liquid. For a 2.09% solution dn/dc was found to be 1.86×10^{-3} at $\lambda = 436 \text{ m}\mu$, and 1.80×10^{-3} at $\lambda = 546 \text{ m}\mu$.

All centrifuge experiments were carried out in NaCl solutions, in general 0.2 *M*, to depress the electrostatic effects. To obtain different pH values buffer salts were added according to Table I, which also gives the results.

Table I. *Sedimentation velocity measurements*

Exp. no.	Salts	Total salt molarity	pH	Protein con- centration, %	s_{20}^*
3	NaCl	0.18	—	0.09	8.29
16	NaCl	0.20	—	0.51	7.91
2	NaCl	0.18	—	0.54	7.85
1	NaCl	0.20	—	1.03	7.93
18	NaCl	0.23	—	1.49	7.64
9	NaCl	0.20	—	2.04	7.52
6	NaCl	0.20	—	6.0	5.81
7	NaCl, NaOAc, HOAc	0.25	4.5	0.85	—†
19	NaCl, NaOAc, HOAc	0.25	4.6	1.0	—†
13	NaCl, NaOAc, HOAc	0.30	4.6	1.05	8.1, 2.6†
5	NaCl, NaOAc, HOAc	0.25	5.0	0.99	7.99
4	NaCl, Na ₂ HPO ₄ , KH ₂ PO ₄	0.24	6.0	1.04	7.97
8	NaCl, Na ₂ HPO ₄ , KH ₂ PO ₄	0.25	8.0	1.06	8.13
10	NaCl, Na ₂ B ₄ O ₇ , KH ₂ PO ₄	0.25	9.0	0.95	7.83
11	NaCl, Na ₂ B ₄ O ₇ , Na ₂ CO ₃	0.25	9.5	1.08	7.61
12	NaCl, Na ₂ HPO ₄ , NaOH	0.25	10.5	1.01	6.88
14	NaCl, NaOH	0.24	12	0.98	1.56†

* Sedimentation constants are given in units of 10^{-13} .

† Many dissociation products of different sizes.

‡ Probably homogeneous.

The figures given in the table for concentrations are analytically determined. (The solutions were weighed out from a stock solution, the concentration of which was determined by evaporation to dryness.) The concentrations were

¹ After this paper had been submitted an article was published by Baranowski (*Hoppe-Seyl. Z.* 1939, 260, 43) in which mention is made of some of our results.

also calculated refractometrically from the sedimentation diagrams, and agreed within a few per cent. with those analytically found except in the runs at pH 4.5–4.6, where the calculated values were definitely lower.

The sedimentation constant depends very little on concentration in the range 0–2 %, although there was a small decrease with increasing concentration. The average s_{20} is 7.86.

Between pH 5 and about 9.5 the sedimentation constant is independent of acidity. The diagrams show only one peak, most probably representing a homogeneous substance. Above and below this range the molecules are not stable. At pH 4.5 a number of different molecular species exist, as revealed by the several peaks in the sedimentation diagram. The same phenomenon was also observed by Deuticke [1934] in his investigation on muscle proteins. The lower limit of the pH stability range is just below 5, as indicated by the fact that in one experiment at pH 4.6 a main component with $s_{20}=8.1$ was obtained, obviously unchanged myogen. The upper limit of the stability range is not so well defined. Deuticke found reduced stability above pH 6.5, but we have not observed any decrease in s_{20} or any inhomogeneity below pH about 9.5.

Diffusion measurements were carried out according to Lamm's [1937] method. The curves obtained were all symmetrical and almost exactly ideal distribution curves, which supports the assumption that the protein is homogeneous. Table II gives the results.

Table II. *Diffusion measurements*

Exp. no.	Salts	Total salt molarity	Protein concentration, %	Time sec.	D ₂₀ * 	Average
1	NaCl	0.2	0.82	76,020 159,900	4.76 4.68	4.72
2	NaCl	0.2	0.65	78,900 166,080	4.89 4.71	4.80
3	NaCl Na ₂ HPO ₄ , KH ₂ PO ₄	0.25	0.92	86,340 167,520	4.79 4.82	4.81
4	NaCl	0.2	0.27	88,740 145,140	4.90 4.72	4.81
Average						4.78

* Diffusion constants are given in units of 10^{-7} .

The partial specific volume of an impure myogen preparation in water was found by Weber [1927] to be equal to 0.74. For the dialysed salt-free solution of the crystalline myogen used in this study it was measured pycnometrically at 20° and was found to be 0.734 ± 0.002 for a 6.07 % solution and 0.735 ± 0.005 for a 2.09 % solution. It is thus independent of concentration within experimental error, and the value 0.735 was accepted as an average, in good agreement with Weber's result.

The molecular weight of myogen A is calculated to be 150,000, using the formula

$$M = \frac{RT s_{20}}{D_{20}(1 - v\rho)}.$$

The ratio of the observed molecular frictional constant to that of a spherical, unhydrated molecule of the same weight, f/f_0 , is 1.26.

The molecular weight was also determined by the sedimentation equilibrium method, using a 1 % solution buffered at pH 7.0. Two cells were used, at different distances from the centre of rotation, and the equilibrium was established

at 4500 r.p.m. The calculations showed no drift in the molecular weight value along the cells. This is a good proof for homogeneity. The values obtained are given in Table III.

Table III. *Sedimentation equilibrium measurements*

Cell I		Cell II	
Distance from centre of rotation cm.	Mol. wt.	Distance from centre of rotation cm.	Mol. wt.
4.70	139,000	5.45	(154,000)
4.75	139,000	5.50	137,000
4.80	139,000	5.55	136,000
4.85	137,000	5.60	137,000
4.90	137,000	5.65	136,000
4.95	136,000	5.70	135,000
5.00	135,000	5.75	134,000
5.05	134,000	5.80	133,000
5.10	134,000	5.85	133,000
5.15	134,000	5.90	132,000
Average	136,000		135,000

The difference between these values and the one obtained by the sedimentation-diffusion method may possibly be explained by experimental errors which cause large absolute errors in the equilibrium method. The value 150,000 is certainly more reliable.

DISCUSSION

The crystalline myogen A is certainly identical with the fastest-sedimenting component found by Deuticke [1934] in extracts from rabbit muscle. He reported a sedimentation constant of 7.70, which is a little lower than that found for crystalline myogen A. The difference may be explained by the fact that Deuticke always had slower-sedimenting molecules present, causing an increase of the viscosity of the medium in which the myogen sedimented. Deuticke also reported a sedimentation constant of the same order of magnitude for a protein in frog muscle extract.

Stöver [1933] has made osmotic measurements on impure myogen and reported a molecular weight of 81,000. The ultracentrifugal methods are certainly more accurate. Stöver found dissociation in urea and association in thiocyanate solutions. In 6*M* urea we found the sedimentation constant to be 1.9 and the diffusion constant 2.4. The myogen was not quite homogeneous in this medium, and the values given are averages. Assuming that the partial specific volume is not changed, a molecular weight of 72,000 is obtained. It is therefore probable that urea splits the molecules into halves. Stöver's figures also indicate such a splitting. Ammonium thiocyanate caused precipitation of most of the protein. Ultracentrifugal analysis showed that the substance left in solution was very heterogeneous with respect to molecular weight, and both association and dissociation seemed to have occurred.

It is of interest to note that myogen, the albumin of the muscle, has about the double molecular weight of serum albumin [70,200, Svedberg, 1939], and that myogen can be split by urea into molecules of this size. It is obvious that Svedberg's multiple rule [1939] is applicable. In the same multiple group of molecular weights ($8 \times 17,600$) are hitherto known only proteins of the globulin type (serum globulins, antipneumococcus serum globulins, and a phycocyan dissociation component). All these proteins have a molar frictional ratio f/f_0 of 1.4–1.5, which is definitely higher than that of myogen, 1.26. The myogen

molecule is therefore more symmetrical or less hydrated than the globulins. Quite possibly both these effects are involved.

The frictional ratio of the myogen molecules split by urea is 3.2, indicating a much more extended shape of the particles. Possibly some intramolecular linkages are split, permitting some sort of unfolding of the molecules.

SUMMARY

1. The following physico-chemical constants for the crystalline myogen A from rabbit muscle were determined:

$$dn/dc = 1.86 \times 10^{-3} \text{ for } \lambda = 436 \text{ m}\mu, \text{ and } 1.80 \times 10^{-3} \text{ for } \lambda = 546 \text{ m}\mu.$$

$$s_{20} = 7.86.$$

$$D_{20} = 4.78.$$

$$V = 0.735.$$

Mol. wt. = 150,000 (136,000 by the sedimentation equilibrium method).

$$f/f_0 = 1.26.$$

2. By different methods of observation (sedimentation, diffusion, sedimentation equilibrium) the protein was found to be homogeneous with regard to molecular weight.

3. Within a pH range from 5 to about 9.5 the molecule is stable. Outside these limits dissociation occurs.

4. Concentrated urea solutions cause dissociation of the molecules into halves.

5. The molecular weight of myogen A falls into the Svedberg multiple system.

The author wishes to thank Prof. Svedberg for the privilege of working in his laboratory and for his kind interest in the investigation. Thanks are due also to Prof. J. K. Parnas, who suggested the problem, and to Dr T. Baranowski, who prepared and supplied the protein. The expenses connected with the work were defrayed by grants from the Rockefeller and Wallenberg Foundations.

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CLXV. METHODS FOR ASSESSING THE LEVEL OF NUTRITION. A CARBOHYDRATE TOLERANCE TEST FOR VITAMIN B₁¹

I. EXPERIMENTS WITH RATS

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IN current nutritional research it is a matter of importance that methods should be made available for detecting the presence of partial or latent deficiencies in the human subject—in other words, methods for assessing the “level of nutrition”. Several such tests have been elaborated during the past few years, including the urinary excretion (“saturation”) test for vitamin C and the dark-adaptation test for vitamin A. For vitamin B₁ the only method so far at the disposal of the investigator has been the test for urinary excretion [Harris & Leong, 1936; Harris *et al.* 1938]. Using this method it was shown that in normal subjects significant amounts of vitamin B₁ are excreted in the urine (the output being related quantitatively to the past intake), that in beri-beri the excretion is negligible, that in “conditioned” polyneuritis (as associated for example with alcoholism, pregnancy, gastro-intestinal obstruction or faulty absorption) there is a similar deficit in the excretion of vitamin B₁, and that in hospitalized patients the excretion is generally low, as a result no doubt of such factors as anorexia, restricted diets, increased metabolism resulting from pyrexia, and other causes [Harris *et al.* 1938]. Although it seems established that the urine test gives a reliable indication of the reserves of a subject in vitamin B₁, and a helpful simplification of the technique is now offered by the proposed substitution of chemical procedures in place of the original biological method of assay [cf. Westenbrink & Goudsmit, 1937, 1, 2; Wang & Harris, 1939], it is still desirable to explore the feasibility of devising alternative methods which might be used alongside the urine test, either for purposes of comparison or as an additional check.

The most likely line of approach seemed to be in the direction of carbohydrate metabolism. It is well known that in experimental animals suffering from beri-beri abnormal quantities of pyruvic and lactic acid accumulate in various tissues and body fluids (for bibliography, see below). Platt & Lu [1936] have reported that in advanced beri-beri in human beings an increased concentration of pyruvic acid is present in the blood and urine; unfortunately, however, the fact that in mild degrees of deficiency little pyruvic acid could be detected seemed to limit the possibility of applying this finding in assessing the level of nutrition. However, we thought it worth while to ascertain whether with intakes of vitamin B₁ only slightly below the optimum there might be some latent diminution in carbohydrate tolerance which could be made apparent by “loading tests” with the products of intermediate metabolism.

¹ Communicated to the Biochemical Society, 9 December 1938 [Banerji & Harris, 1938].

To examine the possibilities of the technique, exploratory experiments have been undertaken in the first place with rats. The results now to be reported show: (1) that an abnormal excretion of pyruvic acid (or bisulphite-binding substances) can be detected in minor degrees of deficiency, and that the amount excreted is graded in proportion to the extent of the deficiency; (2) that administration of lactate serves to accentuate the abnormality; (3) that graded responses are brought about after treatment with graded curative doses of the vitamin.

Preliminary clinical tests indicate that when working with human beings it may be necessary to modify the details of the procedure in some respects, and in the present paper we shall therefore restrict our attention for the most part to the experiments with rats.

Earlier literature on vitamin B₁ and carbohydrate metabolism. The suggestion of Funk [1919] that vitamin B₁ is concerned in carbohydrate metabolism gained support when it was proved that beri-beri in experimental animals was marked by a rise in the level of the lactic acid in their blood or urine [Collazo, 1922; 1923; Bickel, 1924; 1925], liver [Juno, 1926], muscles [Pugliese, 1928; 1929], or brain [Kinnersley & Peters, 1929]. Hayasaka and his colleagues in Japan showed that in human beings suffering from beri-beri the lactic acid in the blood persisted for a long time at a high level following exercise [Inawashiro & Hayasaka, 1928; Hayasaka, 1930, 1, 2]; similar observations were made with dogs [Hayasaka, 1930, 3]. Pyruvic acid in the blood of pigeons and rats with beri-beri was reported by Thompson & Johnson [1934; 1935] also by Johnson [1936]; and independently Platt & Lu [1935; 1936] made the observation, already referred to above, of the presence of pyruvic acid in the blood, cerebro-spinal fluid and urine of patients suffering from the advanced stages of beri-beri. For fuller bibliography, see Harris [1938].

EXPERIMENTAL

Principle of titration method. For the titration of bisulphite-binding substances (B.B.S.) in the urine we have used the technique of Clift & Cook [1932]. Theoretically this gives a measure of the total amount of aldehydic plus certain ketonic substances. There is evidence that the greater part of the titre is in fact due to pyruvic acid (see below, p. 1354), but it is recognized that the method is not a specific one for pyruvic acid alone. The principle of the titration is as follows. Excess of sodium bisulphite is first added to the acidified urine so as to bind aldehyde and ketone groups. The portion of the bisulphite remaining uncombined is then "killed" by titration with standard iodine solution. Finally, the bound bisulphite is set free by the addition of alkali and its amount is estimated by a second titration with iodine.

Details of titration

The urine is adjusted to a pH value between 2 and 4 and is centrifuged if there is any precipitate. 1 ml. of the clear acidified urine is taken, 5 ml. of a 1% solution of sodium bisulphite are added and the mixture is allowed to stand for 15 min. 1 ml. of 1% solution of boiled starch is then added as indicator and the excess of bisulphite neutralized with iodine. For this purpose 0.1 N iodine is first run in until the end-point has been nearly approached and then the end-point is more carefully reached with 0.01 N iodine, a light blue colour being taken as marking the end of the titration. 5 ml. of a saturated solution of sodium bicarbonate are now added and a second titration is carried out with 0.01 N iodine, the end-point being the same light blue colour as in the first titration. The coloration disappears in a short time, especially when the flask is shaken to an undue extent. The reading is taken when the light blue colour persists for from 10 to 15 sec. The number of ml. of the 0.01 N iodine so required

in the second titration is noted and the values are expressed in terms of pyruvic acid:

1 ml. of 0.01 N I_2 = 0.44 mg. pyruvic acid.

"*Bisulphite titre.*" In three of our preliminary tests (Exps. 1-3 in this paper), the urine was not made strongly acid before the initial addition of bisulphite, the binding being allowed to take place at the natural pH value of the urine. It is evident from the results (see p. 1350), that this slight variation in the conditions of the procedure does not materially affect the conclusions reached. But as the quantitative values may possibly vary slightly according to the technique followed, we have thought it best to refer to figures obtained without previous acidification of the urine as "bisulphite titres", and when, as in all later work, the urine was first made acid we retain the standard expression "bisulphite-binding substances". According to Clift & Cook [1932] the capacity of glucose to bind bisulphite is lowest at a pH value between 2 and 4; hence the influence of glucose, if any, on the titre has been minimized in the later experiments.

PART I. EXCRETION OF B.B.S. WITH DIFFERENT LEVELS OF INTAKE OF VITAMIN B_1

In the first four experiments to be described we estimated the excretion of B.B.S. (or "bisulphite titre") over long periods of time in rats receiving adequate amounts of vitamin B_1 as compared with others receiving no vitamin or receiving various suboptimal amounts. As the conditions have varied a little from one experiment to the next, the experiments are best described separately.

Exp. 1. Comparison of normal and deficient rats

The object here was to measure the change in the bisulphite titre during the course of development of avitaminosis- B_1 . Later the conditions were reversed, the avitaminous rats being given the vitamin and the positive group being deprived of it.

Experimental details. Three young rats weighing 60-70 g. were placed on a standard basal diet [Birch & Harris, 1934] free from vitamin B_1 . Three similar rats taken as positive controls were placed on the same diet supplemented with vitamin B_1 , 5 I.U. of crystalline aneurin being allowed, mixed with the diet, on 4 days out of every 5. The rats were isolated in separate cages¹ except on every 5th day, when they were placed three in a group in metabolism cages for the collection of their urine, and on this day no vitamin B_1 was given. It happened that in this test the positive group was all male and the negative all female: later experiments indicate that the result does not depend on the sex.

Result. As seen from Fig. 1 the "bisulphite titre" rose slowly with the development of the avitaminosis, until on the 30th day the average figure was about 27 mg. per rat in contrast with the normal of about 3-7 mg. When vitamin B_1 was then given, the deficient animals began to recover their lost weight and within about 10 days their bisulphite titre had also sunk to within the normal range.

Exp. 2. Comparison of normal and deficient rats; confirmatory test

Experimental details. Conditions were the same as in Exp. 1 except that the 3 rats of each group were kept housed together throughout, including each 5th day when they were placed in the metabolism cages. The rats in the positive

¹ Isolation is a very important precaution, to prevent the danger of coprophagy. A coprophagous rat will continue to thrive in the absence of vitamin B_1 and so give misleading results. In later experiments we have found it advisable to keep the rats isolated even when they are placed in the metabolism cages.

group received 15 I.U. of crystalline vitamin B₁ incorporated daily in their mixed diet, so that their individual intake was approximately 5 I.U. per rat.

Result. As in Exp. 1, the development of avitaminosis-B₁ was associated with a slow rise in the bisulphite titre (see Fig. 2).

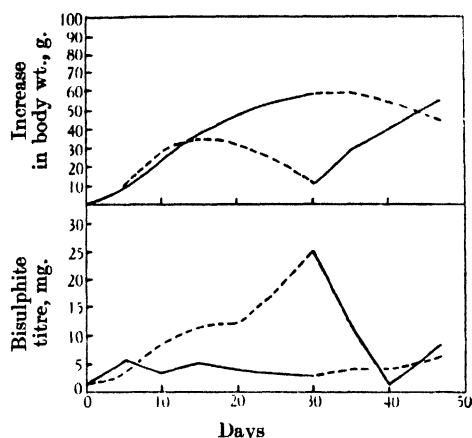


Fig. 1.

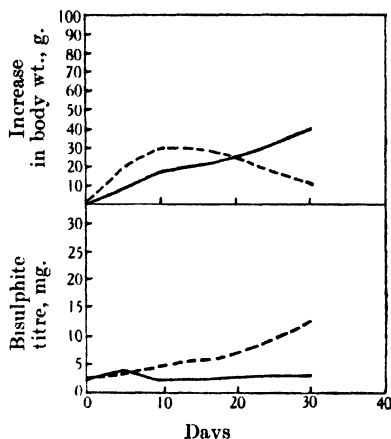


Fig. 2.

Fig. 1. Increase in "bisulphite titre" (p. 1448) in rats deprived of vitamin B₁, as compared with a normal group receiving it. On the 30th day the conditions were reversed. Each curve represents the average of 3 rats, the bisulphite titre being from a mixed specimen of urine.

— With vitamin B₁. ---- Without vitamin B₁.

Fig. 2. Increase in "bisulphite titre" in deficient rats, confirmatory experiment. Values for body weight and bisulphite titre show the average of the group of 3 rats.

— With vitamin B₁. ---- Without vitamin B₁.

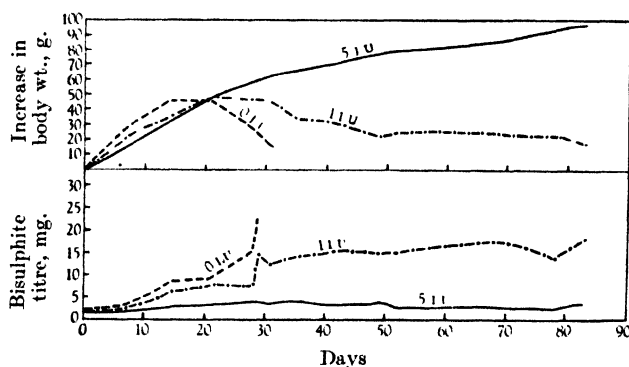


Fig. 3. Growth curves and bisulphite titres for rats receiving (a) zero, (b) partially adequate, and (c) high intakes of vitamin B₁. Average curves, bisulphite titre from combined urine.

— Rats receiving 5 I.U. vitamin B₁ daily. ---- Rats receiving 1 I.U. vitamin B₁ daily.
 Rats receiving no vitamin B₁.

Exp. 3. Effect of partial deficiency

In this experiment, in addition to the positive and negative groups there was a third group of rats receiving a restricted intake of the vitamin—i.e. enough to procure prolonged survival but insufficient for growth at the optimal rate.

Experimental details. The rats were all males and were isolated except on every 7th day when the 3 rats in each group were placed together in the same metabolism cage for the collection of urine. The first group was given no vitamin B₁, the second group (partial deficiency) 1 i.u. daily and the third (normal controls) 5 i.u. daily. The vitamin was given by intraperitoneal injection ("Benerva" brand, Roche Products).

Result. The experimental data (Fig. 3) show that just as the growth curve is related to the intake of the vitamin, so also is the bisulphite titre. With rats receiving no vitamin B₁ death occurred between the 30th and 35th days, and the bisulphite titre climbed to a maximum of about 25 mg. For the rats having 1 i.u. daily, the bisulphite titre rose slowly until about the 5th week and then stayed almost steady at a level of about 13–17 mg. till the 83rd day when the experiment was discontinued. It seems clear that the first 5 weeks represent the depletion period, and thereafter the excretion is characteristic of the particular level of deficiency (cf. Figs. 4–6). The group of rats having a nearly optimum

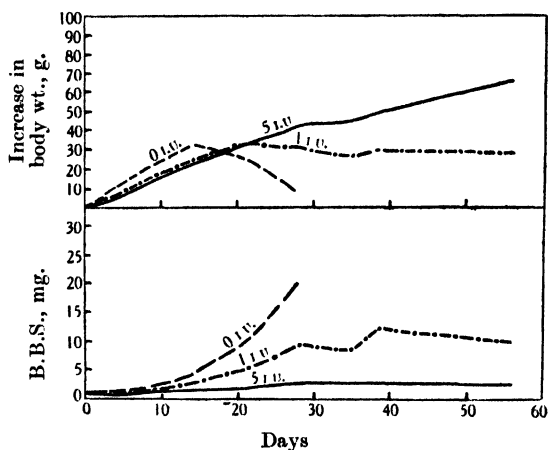


Fig. 4. Proportionality between degree of deficiency and B.B.S. Average curves.

— Rats receiving 5 i.u. vitamin B₁ daily. — — — Rats receiving 1 i.u. vitamin B₁ daily.
- · - · - Rats receiving no vitamin B₁.

allowance of 5 i.u. showed no change in their bisulphite titre, which remained steady throughout the experiment at about 3 mg. per rat per day.

Exp. 4. Effect of partial deficiency; confirmatory experiment

The conditions in this test were the same as in Exp. 3 except that all rats were completely isolated, even on the days when they were kept in the metabolism cages. The urine of each rat was collected separately but was united with that from the other two in the same group for estimation. In this and all later experiments the urine was acidified (pH 2–4) before the addition of bisulphite and the titration results are therefore described as B.B.S. and not "bisulphite titres" (p. 1348).

Result. The results (Fig. 4) agree with those of Exp. 3 in showing that the level of excretion is graded in proportion to the extent of the deficiency. The actual values for B.B.S. in Exp. 4 are in excellent accord numerically with those for "bisulphite titre" in Exp. 3.

PART II. TOLERANCE TESTS WITH PARTIAL DEFICIENCIES

In further experiments we have investigated the possibility of accentuating the abnormal excretion of pyruvic acid (or other bisulphite-binding substances) seen in mild deficiency by "loading" the animal with a related intermediate metabolite, namely sodium lactate.

Exp. 5. Effect of "loading" with lactate at various levels of deficiency

Four levels of intake of vitamin B₁ were given, viz. complete deficiency (0 i.u. daily), moderate degree of deficiency (1 i.u.), intake approaching the optimum (2 i.u.) and presumed full adequacy (10 i.u.). At each level the identical dose of sodium lactate was given daily and the effect on the excretion of B.B.S. measured.

Experimental details. Twenty-four rats in all were used, all males, and were divided into 4 sets corresponding with the 4 levels of intake of vitamin B₁. Each set was further subdivided into 2 groups of 3 rats each, the first group receiving the sodium lactate, the second being the corresponding control without the lactate. The vitamin B₁ was injected intraperitoneally and the sodium lactate was given throughout the experiment as 0.2 ml. of a 50% solution (B.D.H.) mixed with the diet. Rats were isolated both when in their ordinary cages and when transferred for 24 hr. to the metabolism cages at intervals of every 7th day.

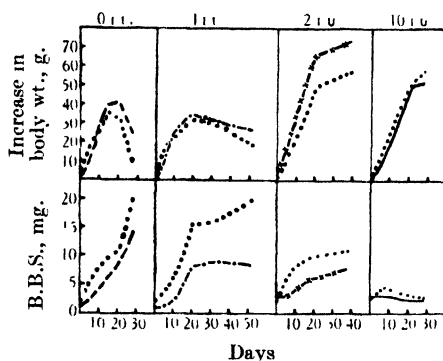


Fig. 5. Effect of sodium lactate in accentuating excretion of B.B.S. in partial deficiency of vitamin B₁. The dotted line shows the influence of the administration of lactate (0.1 g. daily) at four different levels of intake of the vitamin (viz. 0, 1, 2 and 10 i.u. per rat per day). The effect is made most apparent with prolonged partial deficiency, e.g. at the intake of 1 i.u. daily. Curves represent the averages of 3 animals in each group.

..... With lactate added to the diet. - - - - - } Without lactate.
 - x - - - - - }

Result. In the deficient animals the dosing with lactate had the desired effect of accentuating the excretion of B.B.S. (Fig. 5). Particularly with a mild degree of deficiency (1 i.u. daily) was this effect made most usefully apparent. With a severer degree of deficiency (0 i.u.), on the other hand, there already occurred a good rise in B.B.S. during the relatively short survival period without the need for accentuating it by dosing with the lactate. As the intake approaches adequacy (2 i.u.) the animal becomes increasingly tolerant to the effect of the lactate, until, when the supposed optimum intake (10 i.u.) is reached, the lactate is virtually without effect.

The deleterious effect of the lactate on the deficient or partially deficient animal, but not on the normal animal receiving ample supplies of vitamin B₁, is apparent also in the growth curves. That is to say, at levels of 0, 1 and even 2 I.U., growth was retarded by the addition of lactate, but not at a level of 10 I.U.

It may be concluded that rats become increasingly sensitive to lactate according to the degree of the deficiency.

PART III. CURATIVE ACTION OF GRADED DOSES OF VITAMIN B₁

If a carbohydrate tolerance test of the kind we have been describing is to be applied to human beings it is obvious that it should always be controlled by ascertaining whether the administration of vitamin B₁ to the subject under test corrects or does not correct the error in question. Otherwise the possibility would always remain that the defect in metabolism might be due to some other abnormality and not specifically to a deficiency of vitamin B₁.

During the course of Exp. 1 (p. 1348) it was already shown that the continuous administration of vitamin B₁ to avitaminous rats for a period of about a week slowly brought down the bisulphite titre from a high value of around 27 mg. to the normal figure of 4 mg. In the work now to be reported our object was to examine the effect of single graded doses of vitamin B₁ on the deficient animal, so as to discover whether the effect was proportional to the amount given.

Exp. 6. Effect of single doses of vitamin B₁; preliminary experiment

In a preliminary experiment (Fig. 6), 3 rats after being on a deficient diet for about 3½ weeks, during which time their B.B.S. value had steadily risen, were injected intraperitoneally each with a dose of 5 I.U. The result was a slight but distinct drop in the titre, persisting for a day or two. With a somewhat larger dose, 7 I.U., the effect was appreciably greater.

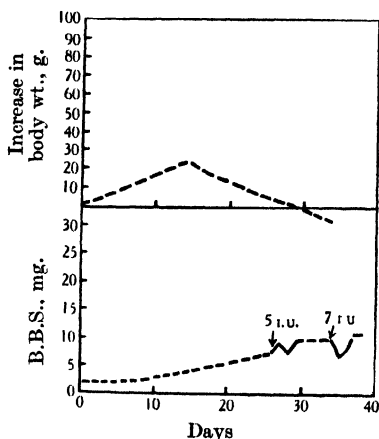


Fig. 6.

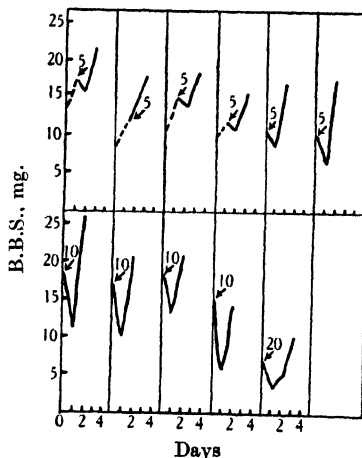


Fig. 7.

Fig. 6. Temporary drops in B.B.S. curve, produced by single doses of vitamin B₁. 5 and 7 I.U. were given at the first and second arrows respectively. Averages for 3 rats; B.B.S. determinations made on mixed specimens of urine.

Fig. 7. Relative effects of different test-doses of vitamin B₁. Vitamin given at arrow. Top line, test doses of 5 I.U.; Bottom line, 10 I.U.; one instance, 20 I.U. Each curve represents the average for 3 rats, the specimens of urine being combined.

Having found the magnitude of the response to be expected we were able to undertake more detailed systematic studies, covering a wide range of graded doses.

Exp. 7. Response to graded doses of vitamin B₁

In this experiment, single test-doses of 5, 10 and 20 I.U. were given to a large series of rats.

Experimental details. The rats were kept isolated throughout, but the urines from each batch of 3 rats similarly dosed were always combined for estimation of B.B.S. In order to increase the accuracy of the estimation, the insides of the funnels of the metabolism cages were rinsed out with water and the washings added to the urine.

Result. In Fig. 7 it will be seen that the magnitude of the effect (as measured by the time elapsing before the B.B.S. returns to the same level as when the vitamin was injected) is graded in proportion to the dose given. A dose of 10 I.U. gives a greater response than one of 5, and 20 a still greater response.

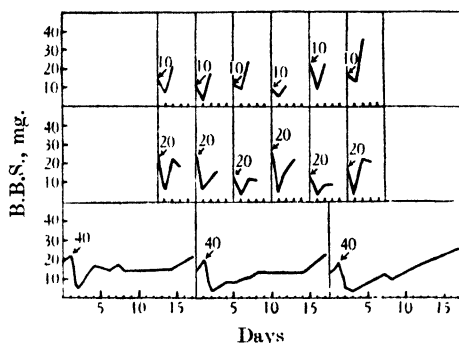


Fig. 8. Proportionality between dose of vitamin and duration of response. Top line, doses of 10 I.U. given at arrow. Middle line, 20 I.U. Bottom line, 40 I.U. Each curve represents the result from an individual rat (cf. Fig. 7).

Exp. 8. Response to graded doses; confirmatory experiment

This experiment (Fig. 8) resembles Exp. 7, except that a higher range of doses was tested, namely 10, 20 and 40 I.U. Also, the urine of each individual rat was tested separately instead of the combined urine of 3 rats.

Result. As in Exp. 7, it is clearly shown that the duration of the response increases regularly with the size of the dose. There is good agreement between individual animals receiving the same dose (Fig. 8).

DISCUSSION

As already indicated three principal conclusions stand out from the experimental work. (1) The excretion of B.B.S. increases with increasing degrees of deficiency. (2) With mild degrees of deficiency the latent abnormality in carbohydrate metabolism can be made more clearly apparent by tolerance tests with lactate. (3) Graded responses follow graded doses of the vitamin.

Suggested use for vitamin estimations. The possibility of applying these observations for assay work with rats is obvious. Two types of procedure might be considered, either prophylactic experiments in which the rise of B.B.S. is measured with different allowances of the food under investigation, or curative experiments in which the effect is tested of single or daily repeated doses in

procuring a fall of the high B.B.S. The latter alternative might be preferred as necessitating a less lengthy test, provided, that is, that an animal is kept available which has been already "run out". Since a relatively large single dose of the vitamin is needed to bring about a "cure" of any duration, as measured by the B.B.S. figure, the more convenient procedure may be to give the supplement daily for some days and measure the rate of fall. It is important to control the intake of carbohydrate and fat. Further tests of this kind are in progress.

Applications to human beings. Preliminary observations on human beings indicate that some of the highest B.B.S. values so far noted have been associated with a low vitamin B₁ content in the urine, and sometimes with neuritic symptoms. Especially in pregnancy have these low values for vitamin B₁ and high values for B.B.S. been seen. This finding seems of significance, especially when it is recalled that the needs for vitamin B₁ are known to be increased during pregnancy and lactation, and that cases of neuritis in pregnancy have previously been found [Harris *et al.* 1938] to be marked by low reserves of vitamin B₁ (i.e. to be a "conditioned deficiency" state).

By courtesy of Mr Y. L. Wang and Dr J. Yudkin we are able to refer here to tests now in progress on a group of 3 volunteers placed on a beri-beri-producing diet (polished rice, autoclaved milk etc.). The B.B.S. titre of their urine began to show a steady rise almost immediately, and after about a week had already reached values as high as 3-4 times the initial values (470-670 mg. per day in place of the original 140-190), notwithstanding the fact that vitamin B₁ was still being excreted at a moderately high level. But at the same time, it is important to recognize that figures for normal subjects may sometimes cover a very considerable range of variations, approaching on occasions values as high as those found in patients presumed to be low in vitamin B₁. It is clear that variable factors, particularly the intake of carbohydrate and fat in the diet, play an important part, and even with a deficiency of the vitamin high B.B.S. values may not be recorded unless extra carbohydrates (or suitable intermediate metabolites) are given to balance the lowered consumption of food. Obviously much more work remains to be done before we can speak with confidence of any normal range of standards for human beings, or indeed specify the most suitable conditions for assessing the deficiency by measurement of the abnormal carbohydrate metabolism. Tests are in progress in which lactate as well as pyruvate and other substances are being measured in various body fluids, both after test doses of either of these substances or of other metabolites, and under various controlled conditions (e.g. regulation of amount of exercise and provision of standardized diet of specified carbohydrate and fat content).

Nature of bisulphite-binding substances in urine. There seems little doubt that the major part of the B.B.S. titre for avitaminous urine represents pyruvic acid, although, as previously mentioned, other substances may theoretically be included such as methylglyoxal, α -ketoglutaric acid, or indeed any molecule containing aldehydic or certain ketonic groups (or both). The discussion of this point will be deferred to a later paper, but in the meantime it may be worth emphasizing that in conditions such as diabetes where a ketonuria is present, it is essential that the results be controlled by observing whether or not a high bisulphite titre responds to dosing with vitamin B₁.

SUMMARY

The possibility has been investigated of assessing partial degrees of deficiency of vitamin B₁ by measurement of latent defects in the carbohydrate metabolism.

In exploratory experiments, rats have been restricted for prolonged periods to various graded suboptimal intakes of vitamin B₁. It has been shown that:

(1) The excretion of bisulphite-binding substances (B.B.S.) in the urine reached levels proportional to the extent of the deficiency of the vitamin (Figs. 1-4).

(2) The administration of sodium lactate intensified the excretion of B.B.S. at each level of deficiency or partial deficiency, so that minor deficiencies could be more readily made apparent (Fig. 5).

(3) The administration of a single dose of vitamin B₁ gave a temporary respite from this abnormal excretion of B.B.S. proportional to the size of the dose so given (Figs. 6-8).

(4) The optimum requirement for vitamin B₁ needed to secure normal carbohydrate metabolism (or maximum tolerance) was many times the ordinary bare protective dose.

Proposed applications of this technique are: (1) in rat tests for the assay of vitamin B₁ in foods, by analysing the rat's urine; (2) for assessing the level of nutrition of human subjects, as an alternative to measurements of the vitamin B₁ in urine. Preliminary tests on human subjects indicate that an increase in B.B.S. can be produced experimentally by a diet low in vitamin B₁ and that high values frequently accompany subnormal excretion of vitamin B₁ (e.g. during pregnancy). Further work is needed, however, to define quantitatively the most accurate conditions for applying the carbohydrate tolerance test to human beings so as to control other variables, e.g. basal diet, exercise. The results have always to be checked by giving vitamin B₁ to prove whether the abnormal carbohydrate intolerance is the result of a specific vitamin B₁ deficiency.

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CLXVI. METHODS FOR ASSESSING THE LEVEL OF NUTRITION OF THE HUMAN SUBJECT. ESTIMATION OF VITAMIN B₁ IN URINE BY THE THIOCHROME TEST¹

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IN earlier work [Harris & Leong, 1936; Harris *et al.* 1938] it was shown that the extent of the body's "reserves" of vitamin B₁ might be measured by means of a test on the urine, similar in principle to that previously introduced for vitamin C [Harris *et al.* 1933; Harris & Ray, 1935]. The urinary output was found to depend on the past intake, being negligible in beri-beri or in "conditioned" deficiencies of vitamin B₁ and being lowered in certain other abnormal states.

To simplify the procedure, several investigators [e.g. Westenbrink & Goudsmit, 1937, 1, 2; 1938; Karrer, 1937; Ritsert, 1938; Marrack & Höllering, 1939] have proposed to substitute the thiochrome reaction of Jansen [1936] for the biological (bradycardia) method of assay originally employed. Various alternative techniques for the thiochrome test have been recommended by these different workers and these may give rather widely divergent results. Since also the accuracy of the thiochrome method as applied to urine has been questioned [e.g. Pyke, 1939; cf. also Henry *et al.* 1938], it seemed advisable to undertake a thorough study of the details of the procedure, and at the same time (and perhaps even more important) to standardize the results obtained against direct biological assays.

The principal difficulty which we have encountered has been the presence in urine of various non-specific substances which may seriously interfere in the measurement of fluorescence, upon which the thiochrome reaction depends. To overcome this and other sources of error we are recommending in the present paper a number of modifications in the technique, which have the advantage also of improving the simplicity and sensitivity of the method. As will be shown, the results given by this procedure have been proved to be accurate when checked against biological tests, and vitamin B₁ added to urine has been quantitatively recovered. By the method described tests may be completed on a batch of, say, three to six urines within about an hour; the error is ordinarily less than about 10 %, and a specimen of not more than about 5 ml. suffices for the examination.

Principle of the thiochrome method

The principle of the thiochrome reaction is to transform the vitamin B₁ into its fluorescent derivative, thiochrome, by means of a mild alkaline oxidation with ferricyanide, and to measure the degree of the fluorescence against some suitable standard.

According to the procedure of Westenbrink & Goudsmit [1937, 1, 2] the vitamin B₁ is first adsorbed on franconite and the fluorescence is subsequently measured by a photoelectric fluorimeter. The procedure of Hennessy & Cerecedo [1939] is similar except that the vitamin B₁ is

¹ Communicated to the Biochemical Society, 6 May 1939 [Wang & Harris, 1939].

adsorbed on synthetic zeolite, while Marrack & Höllering [1939] adsorb on franconite but measure the fluorescence by a comparison with the naked eye. Ritsert [1938] omits altogether the preliminary adsorption, but provides for the large blank-correction either by (a) his "compensation method", or (b) by the so-called "automatic equalization". Like Ritsert we consider it of great importance to pay attention to the presence of interfering substances, but our procedure differs essentially from his in that our aim has been to concentrate as far as possible on their removal from the sphere of the reaction rather than to attempt to allow for them by a very large and sometimes uncertain blank correction (see below, p. 1359).

Examination for possible sources of error

In a series of preliminary tests the various possible sources of error were examined. The most important of these were found to include the following:

- (1) Incomplete removal of the vitamin on adsorption.
- (2) Interference from non-specific fluorescing substances present in urine.
- (3) Interference from reducing substances other than the vitamin.
- (4) Interference from pigments.
- (5) Fluorescence caused by use of impure reagents.

These difficulties may be briefly discussed in turn.

(1) *Incomplete adsorption.* While the vitamin may generally be removed nearly quantitatively from such specimens of urine as are rich in it (provided that enough of the adsorbent is used, and the right conditions of pH etc. are maintained), it is more difficult to effect anything approaching complete separation when working with dilute samples (see Table VI). Another objection to the use of adsorption, so far as the thiochrome test is concerned, is that it brings about a concentration from the urine not only of the vitamin itself, but also of other reducing substances which increase the interference at a later stage by requiring the addition of an undue amount of the ferricyanide reagent (see no. 3 below).

(2) *Non-specific fluorescing substances.* We have found that urine itself may contain variable amounts of preformed thiochrome, and this, together with other non-specific fluorescing substances, will be estimated in the final comparison unless steps are taken to obviate this source of error. The non-specific substances produce a yellowish-white fluorescence, in contrast with the bluish or violet fluorescence given by the thiochrome. The presence of the non-specific fluorescing substances seems to affect also the actual colour of the fluorescence given by the thiochrome itself.

(3) *Interference from reducing substances.* Reducing substances other than vitamin B₁ which are present in urine, and which as mentioned under no. 1 above may become concentrated by preliminary adsorption, interfere by making it necessary to add a large excess of ferricyanide to the urine beyond the small amount theoretically needed to oxidize the vitamin B₁ alone. The objection to this large addition of ferricyanide is twofold: (1) it destroys some of the thiochrome produced by the reaction; (2) it has a variable effect on the non-specific fluorescing substances, making it impossible to allow for them accurately in a blank control.

(4) *Interference from pigments.* The presence of natural pigments in the urine may mask the fluorescence of the thiochrome in the final solution to such an extent as to make matching difficult.

(5) *Fluorescence caused by use of impure reagents.* Virtually all the filter papers examined were found to yield fluorescing substances which would interfere with the test. Sodium sulphate, used for drying the solution of thiochrome in isobutanol, may also sometimes be contaminated with fluorescing materials.

Table I outlines the procedure used by past workers, together with the corresponding possible sources of error just referred to which may be expected to interfere with the accuracy of the test under the various conditions.

Table I. *Methods for estimating vitamin B₁ in urine by thiochrome reaction*

Worker	Procedure	Possible sources of error
(1) Westenbrink & Goudsmit [1937, 1, 2]	Adsorb on franconite. Compare by photo- electric fluorimeter	(a) Vitamin B ₁ incompletely ad- sorbed (b) Reducing and colouring sub- stances adsorbed along with vitamin B ₁ , necessitating unduly large addition of K ₃ Fe(CN) ₆ (c) Non-specific fluorescing sub- stances interfere with fluori- meter reading
(2) Karrer [1937]	Adsorb on franconite. Compare by naked eye	Similar to no. 1
(3) Hennessy & Cerecedo [1939]	Adsorb on zeolite. Compare by fluori- meter	Same as no. 1
(4) Marrack & Höllering [1939]	Adsorb on franconite. Compare by naked eye	Probably similar to no. 1
(5) Ritsert [1938]	No preliminary ad- sorption. Compare by naked eye. Blank allowed for (a) by "compensation me- thod", (b) by "auto- matic equalization"	Procedure tedious for method (b), and "blank correction" relatively inaccurate (see below, p. 1359)

Special features of the technique recommended

After numerous trials and control tests a procedure was eventually evolved which seems to overcome these several sources of error, which gives accurate results when tested against biological assays, and which is rapid and simple. The principal points of this procedure include the following:

- (1) No preliminary adsorption.
- (2) Removal of preformed thiochrome and other interfering substances by preliminary extraction of urine with *isobutanol*.
- (3) Regulation of amount of K₃Fe(CN)₆ added.
- (4) Treatment of filter paper and reagents.
- (5) Further removal of interfering substances by washing the final *isobutanol* solution of thiochrome with water.
- (6) Direct visual comparison of fluorescence.
- (7) Allowance in blank for residue of interfering substances not previously removed.

The reasons for these steps may be briefly noted as follows:

(1) *Absence of preliminary adsorption.* As will be clear from the foregoing discussion the advantages of omitting the adsorption, apart from the convenience and great saving of time, are that the danger of loss of vitamin is obviated and there is no undesirable over-concentration of interfering substances.

(2) *Preliminary washing with isobutanol.* With urines containing appreciable amounts of preformed thiochrome or with specimens which are highly pigmented or relatively poor in the vitamin, this procedure significantly improves the accuracy of the method.

(3) *Regulation of $K_3Fe(CN)_6$* . As already explained the addition of excess of ferricyanide interferes with the reliability of the method, and to overcome the error we ascertain in a separate control test what amount is necessary.

(4) *Treatment of filter paper and reagents*. The presence of fluorescing substances in the filter paper may alone give rise to a considerable error if not allowed for.

(5) *Washing of isobutanol layer*. This we regard as an important step, as it removes a large portion of the non-specific fluorescing substances and helps to clear the solution from further traces of pigments. The water also extracts anions, which in certain fluorimetric tests are known to have a variable influence on the degree of fluorescence.

(6) *Visual comparison of fluorescence*. For the sake of simplicity and saving of expense and time, a matching of the end-point by the eye seems to be desirable. The accuracy is not less than with a photoelectric apparatus, and the prior removal of the greater part of the blank enables the matching to be done with accuracy.

(7) *Blank control*. To allow for any residuum of interference a blank correction is introduced. However, the blank is of a small order of magnitude in comparison with the "unknown", instead of being of a large order as when interfering substances are not first removed.

Ritsert (see Table I) proposed to allow for a blank correction either (a) by his "compensation" method or (b) by the so-called "automatic equalization". The former alternative involves the addition of an isobutanol extract of the untreated urine to the control for the final matching; the disadvantage of this procedure seems to be that the blank is excessively large in proportion to the small amount of the unknown and there is also interference from any preformed thiochrome in the urine. The second alternative ("automatic equalization") involves the use of a blank consisting of urine from which the vitamin has been removed by adsorption on franconite. Unfortunately such a procedure may entail a partial and variable removal of the blank fluorescence (so that the extent of the blank cannot be adequately controlled) and also an incomplete removal of the vitamin B_1 itself.

Outline of procedure

The process recommended involves five successive steps. (1) A small volume of urine is washed with an equal bulk of isobutanol. (2) The washed urine is treated with methanol, ferricyanide and NaOH, to convert the vitamin B_1 into thiochrome. (3) The "thiochrome" so formed is extracted with isobutanol. (4) The isobutanol layer is washed with water, cleared with Na_2SO_4 (or ethanol), and a specimen taken for measurement of fluorescence. (5) In the measurement of fluorescence a standard solution of thiochrome is added drop by drop to a blank used as a control, while at the same time an equal volume of isobutanol solvent is added to the unknown, until the fluorescence of the two is matched.

Working details

It is important that strict attention should be paid to procedure if reliable results are to be obtained, and accordingly the working details are given below in full.

Reagents.

$K_3Fe(CN)_6$, 2 % aqueous solution.

NaOH, 30 % aqueous solution.

Methanol.

isoButanol (redistilled).

Na_2SO_4 , anhydrous.

All reagents, particularly the Na_2SO_4 and the redistilled *isobutanol*, should be tested for freedom from fluorescence in blank determinations.

Apparatus.

Ultraviolet mercury-vapour lamp fitted with filter of Wood's glass.

Three glass-stoppered graduated cylinders, 25 ml. capacity.

Centrifuge.

Filter papers, extracted with *isobutanol* for not less than 12 hr. in a Soxhlet apparatus until found to be free from fluorescent substances in blank determinations.

Procedure.

(1) *Preliminary washing.*¹ Measure 5–8 ml. of urine into a 15 ml. graduated centrifuge tube, add an equal volume of *isobutanol* and 2 drops of 10 % HCl. Shake vigorously for 2 min. and centrifuge. Note the final volume of the aqueous layer. Discard the *isobutanol* layer.

(2) *Oxidation.* Measure three 1–2 ml. portions of the extracted urine, depending on the concentration of vitamin B_1 in it, into 3 graduated glass-stoppered cylinders of 25 ml. capacity. Add to each 2 ml. of MeOH. To the first two of these cylinders add 1 ml. of 30 % NaOH and mix. To the first cylinder add 2 % $\text{K}_3\text{Fe}(\text{CN})_6$ from a graduated pipette cautiously drop by drop, shaking between each addition until the colour is just slightly more yellow than in the second cylinder which has no $\text{K}_3\text{Fe}(\text{CN})_6$ and which is to be used subsequently as a control. Avoid an excess of ferricyanide; this is very important, otherwise it will be impossible to get a perfect match of fluorescence. The slight colour should stay for at least half a minute. Note the volume of $\text{K}_3\text{Fe}(\text{CN})_6$ so required. Add exactly the same amount of $\text{K}_3\text{Fe}(\text{CN})_6$ to the third cylinder (i.e. the one without NaOH), mix, and then add 1 ml. of 30 % NaOH. Mix and allow to stand for 1 min.

Cylinder no. 1, used to determine the amount of $\text{K}_3\text{Fe}(\text{CN})_6$ needed, is not further required.

(3) *Extraction of thiochrome.* Add 10 ml. of *isobutanol* both to cylinder no. 2 and to no. 3, shake vigorously for 2 min. and allow the layers to separate.

(4) *Washing and drying.* Cylinders nos. 3 ("unknown", with ferricyanide) and 2 (control, without ferricyanide) are now treated identically as follows. Remove the bottom (aqueous) layer with a long pipette fitted with a rubber teat.² Add 4 ml. of water to each cylinder and shake vigorously for 1 min. Control tests indicate that under these conditions no appreciable quantity of the thiochrome enters the watery phase. Again allow the layers to separate. Make up the *isobutanol* layer to 15 ml. with fresh *isobutanol* and remove it to a dry conical flask. Add gradually approximately 4 g. of anhydrous Na_2SO_4 to each flask, shaking during the addition; filter through the *isobutanol*-extracted filter

¹ For routine determinations this step is not usually necessary, but for accurate work and with certain urines containing large amounts of interfering fluorescing substances it is desirable (and may be essential) to give the urine this preliminary washing. With an occasional specimen a second and even third washing may be necessary. Control tests show that no significant amount of vitamin B_1 is lost in the washing.

² When working with large batches of urines time may be saved by performing the operations in a specially graduated separating funnel, so that the bottom layer can be quickly run off on each occasion and there is no need to transfer the *isobutanol* layer. A battery of such funnels can be used together and shaken simultaneously in a mechanical shaker.

papers into clean dry test tubes¹ and take 10 ml. of the clear filtrates for comparison of fluorescence. (Alternatively, in place of drying with Na_2SO_4 the solution may be clarified by adding 1 ml. of ethanol and the filtration may then be omitted.)

(5) *Comparison of fluorescence.* Place the two test tubes side by side in front of the Wood's glass window of the ultraviolet lamp, inclining them towards the body at an angle of approximately 60° to the horizontal so that the eye can look into the tubes. Add the standard (see below) little by little from a graduated pipette to the control until the fluorescence exactly matches the unknown. Add to the "unknown" an amount of isobutanol equal in volume to the amount of the standard added to the control, so as to make the final volumes in the two tubes the same. The contents of the tubes may be mixed after each addition by pouring backwards and forwards into another dry test tube. From the volume of the standard used, the content of aneurin can be calculated; e.g. if

x = the number of ml. used of standard, having a concentration of y μg . per ml.,

v = the volume of urine used,

V = the total output of urine per 24 hr.

the aneurin content of the urine = $\frac{xy}{10} \times \frac{1}{v}$ μg . in the 24-hr. specimen.

Preparation of standard.

"Benerva" ampoules (manufactured by Roche Products, Ltd.) can be conveniently used as the standard for aneurin. Prepare a solution containing 1–2 μg . per ml. by diluting a measured volume of the original solution with H_2O and adjust it to a pH value of 3. This solution may be kept for 2–3 days in an ice chest. Measure 1 ml. of the standard into a graduated glass-stoppered cylinder and treat it in exactly the same way as for the urine, using one drop only of the 2% $\text{K}_3\text{Fe}(\text{CN})_6$.

Results: normal subjects

As will be seen from Table II, eleven normal subjects have been examined, consisting of laboratory assistants and research workers at Cambridge. The lowest average excretion for any one subject was about 30 I.U. per day and the highest 130 I.U. The usual range was between 50 and 80 I.U.

Results with special cases

Through the kind co-operation of a number of medical practitioners, to whom we wish to express our indebtedness, we have been able to examine the urinary output of a number of patients (Table III). An interesting feature is the lowered excretion noted in several women during and after pregnancy. This result accords with the view that additional vitamin B_1 is needed by women during gestation and lactation, and that their reserves are liable to be depleted to a dangerously low level, either because of the increased demands of the foetus or the sucking child or sometimes as a result of hyperemesis. It is now well recognized that a conditioned deficiency of vitamin B_1 may be the cause of polyneuritis in pregnancy. A significant finding was that the low excretion of vitamin B_1 in the urine of the pregnant women was found, in tests by Mr G. G. Banerji, to be sometimes associated with increased excretion of bisulphite-binding substances [cf. Banerji & Harris, 1938].

¹ Care must be taken to procure test tubes the glass of which is free from fluorescence in the light of the mercury-vapour lamp.

Table II. *Excretion of vitamin B₁ by normal subjects*

Initials	Date	Remarks	Daily excretion of vitamin B ₁ in I.U.		
			Single days	Range	Average
G. G. B.	3. i. 39	Normal diet	65	65- 83	74
	17. i. 39	"	83		
Y. L. W.	14. xii. 38	After test dose	(200)	90-110	105
	12. iv. 39	Normal diet	110		
	2. vi. 39	"	110		
	3. vi. 39	"	110		
	6. vi. 39	"	90		
A. D.	9. ii. 39	Normal diet	47	—	47
	17. ii. 39	"	47		
G. G.	20. xii. 38	Normal diet	90	70- 90	77
	16. ii. 39	"	70		
	17. ii. 39	"	70		
K. R. R.	9. ii. 39	On brown bread	140	71-140	106
	30. iii. 39	On white bread	71		
J. Y.	17. i. 39	Normal diet	68	33- 68	51
	2. vi. 39	"	53		
	3. vi. 39	"	33		
W. D. R.	18. iv. 39	Marmite daily	110	110-160	132
	25. iv. 39	"	160		
	26. iv. 39	"	140		
	27. iv. 39	"	120		
L. J. H.	25. iv. 39	Normal diet	54	53- 65	57
	26. iv. 39	"	53		
	27. iv. 39	"	55		
	28. iv. 39	"	65		
S. A. C.	20. xii. 38	Normal diet	93	35- 93	55
	3. vi. 39	"	36		
	4. vi. 39	"	35		
A. W.	28. iii. 39	Normal diet	33	—	33
S. I.	9. ii. 39	Normal diet	54	—	54

Table III. *Excretion of vitamin B₁ by patients*

Patient's initials (with name of doctor)	Date	Diagnosis or notes	Vitamin B ₁ excreted I.U. in 24 hr.
Mrs P. (Dr J. Yudkin)	9. i. 39	Subacute combined degeneration, pernicious anaemia	15
	10. i. 39		26
— C. (Dr J. Yudkin)	13. iii. 39	Disseminated sclerosis	16
Mrs W.	27. iii. 39	Anorexia, neurasthenia	29
	29. iii. 39		16
Mrs B. (Dr Silberstein)	20. iv. 39	8 months pregnant	39
— S. (Dr Taylor)	21. iv. 39	Peripheral neuritis	26
	24. iv. 39		0
	25. iv. 39		0
Mrs N. (Dr Lawrie)	21. iii. 39	Late pregnancy; toxæmia of preg- nancy. Same after delivery	32
	19. iv. 39		12
	20. iv. 39		16
Mrs W. (Dr Nicholson)	26. v. 39	Anorexia nervosa	19
	27. v. 39		20
W. T. (Dr Nicholson)	8. v. 39	Lassitude	97

The subnormal values found in certain other patients may presumably be attributed to "conditioned" deficiency (or partial deficiency) arising from faulty absorption or utilization, or restricted diet [cf. Harris *et al.* 1938]. The low "reserves" in the patient (Mrs W.) suffering from anorexia nervosa are of interest. Her medical attendant reported that she was suffering from persistent vomiting and taking little nourishment beyond some milk and two slices of bread and butter per day.

Agreement with biological standardization

To test the accuracy of the results obtained in the thiochrome tests, a comprehensive series of biological controls was carried out as follows. A group of 5 specimens of urine was chosen, varying in activity from the very subnormal figure of 16 I.U./24 hr. to the very high one of 304 I.U. The latter represents an excretion after test-dosing (p. 1367).

As will be seen from the results summarized in Table VII and charted in Fig. 1, there is a most satisfactory parallelism between the chemical and biological results, although the specimens of urine cover no less than a twenty-fold range in their activities.

Details of biological test. For protocols of the full experimental details see Tables IV and V. In each instance the vitamin B₁ was adsorbed from a large bulk of the urine by treatment with "Clarit" acid-clay, and the biological tests were carried out by the bradycardia method as

Table IV. *Details of biological standardization. Series I*

Sample	Vitamin B ₁ content of urine, I.U. per day by thiochrome test	No. of I.U. administered per day by thiochrome test	Days, cured	
			Individual animals	Average
Patient (after se)	304	1	0, 2, 1·25, 1·25, 2, 0·5, 0, 3·25	1·3
		2	3, 1·5, 3, 3, 2, 2	2·4
		3	2, 2, 3·5, 2·5, 4, 3·5, 4·5	3·1
Normal con- G. B.	83	1	2·5, 1·5, 1, 1	1·5
		2	2·5, 4, 3·5, 2·5	3·1
		3	3·5, 2, 4, 4	3·5
ional standard	---	1	1, 2, 2, 2·5	1·4
		2	1·5, 4, 1·5, 2, 1	2·0
		3	3, 2·5, 3·5, 5, 4	3·6

Table V. *Details of biological standardization. Series II*

Sample	Vitamin B ₁ content of urine, I.U. per day by thiochrome test	No. of I.U. administered per day by thiochrome test	Days, cured	
			Individual animals	Average
Urine 3. Normal control Y. L. W.	108	1	1·5, 1, 2, 1·5, 2	1·6
		2	4, 2, 2·5, 2, 3	2·7
		3	4, 3, 3·5, 3, 3·5	3·4
Urine 4. Normal control K. R. R.	71	1	0, 2, 2, 2	1·5
		2	3·5, 2·5, 2, 2, 2	2·4
		3	3, 2, 4·5, 3·5	3·3
Urine 5. Patient Mrs W.	16	1	2, 2	2
		2	3·5, 2	2·8
Urine 6. Patient Mrs N. International standard	16 ---	1	0, 1·5, 0·5, 1, 1·5	0·9
		1	2, 2, 2, 2, 0	1·6
		2	2, 2, 5, 2, 2·5	2·7
		3	3, 3·5, 3, 4, 3	3·3

Table VI. *Calculation of biological activities*

Specimen	Vol. urine per day ml.	Vitamin B ₁ excreted per day, by thio- chrome test I.U.	Amount of acid-clay used per 100 ml. urine g.	% vitamin B ₁ adsorbed (thio- chrome analysis)	Dose of adsorbed vitamin B ₁ given (by thio- chrome) I.U.	No. of doses given for each level	Days cured av.	Activity as read from reference curve, I.U.		Vitamin B ₁ found in adsorbate per ml. urine, I.U.		Differ- ence %
								Per dose given	Per unit given	By brady- cardia	By thio- chrome	
Series 1. Patient Mrs P. (after test dose)	1380	304	2.5	96	1	8	1.3	1.1	1.1	0.23		
					2	6	2.4	2.1	1.05	0.22		
					3	7	3.1	2.7	0.9	0.19		
								Av. 1.02	Av. 0.21	0.21		0
Normal control G. G. B.	1380	83	1	72	1	4	1.5	1.3	1.3	0.056		
					2	4	3.1	2.7	1.35	0.058		
					3	4	3.5	3.0	1.0	0.043		
								Av. 1.22	Av. 0.052	0.043		-17
Series 2. Normal control Y. L. W.	850	108	2	76	1	5	1.6	0.9	0.9	0.091		
					2	5	2.7	2.2	1.1	0.106		
					3	5	3.4	3.0	1.0	0.099		
								Av. 1.00	Av. 0.099	0.099		0
Normal control K. R. R.	2520	71	0.7	71	1	4	1.5	0.8	0.8	0.016		
					2	5	2.4	1.8	0.9	0.018		
					3	4	3.3	2.9	0.97	0.019		
								Av. 0.89	Av. 0.0178	0.020		+11
Patient Mrs W.	1200	16	1	50	1	2	2	1.4	1.4	0.009		
					2	2	2.8	2.3	1.15	0.0075		
								Av. 1.28	Av. 0.0083	0.0065		-22

Table VII. *Results of biological standardization*

	Vitamin B ₁ excreted, I.U. per day	
	By thiochrome	By bradycardia
Series 1:		
Patient (after test dose)	304	304
Normal control G. G. B.	83	99
Series 2:		
Normal control Y. L. W.	108	108
Normal control K. R. R.	71	63
Patient Mrs W.	16	20

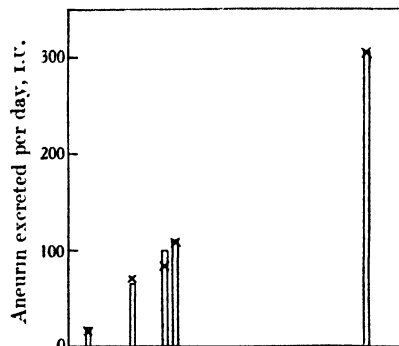


Fig. 1. Agreement between chemical and biological determinations on specimens of urine of varying activity (excretion ranging from 304 to 20 I.U. daily). Columns = biological tests. Crosses = thiochrome estimations.

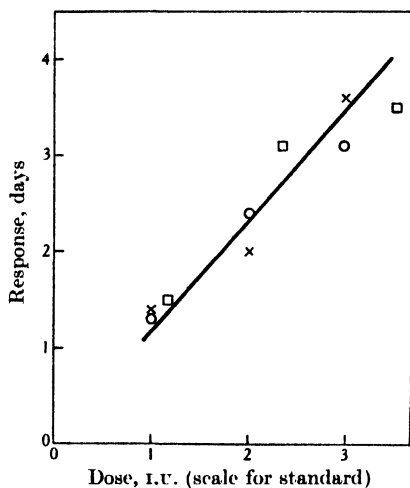


Fig. 2.

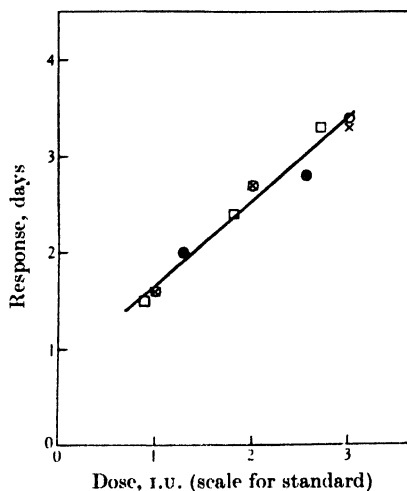


Fig. 3.

Fig. 2. Dose-response curve, series I. \times = international standard. \circ = urine no. 1. \square = urine no. 2. The reference curve is based on the responses with the international standard. The scale of doses for the two specimens of urines is drawn in proportion to the final calculations of their biological activities.

Fig. 3. Dose-response curve, series II. \times = international standard. \circ = urine no. 3. \square = urine no. 4. \bullet = urine no. 5. Reference curve drawn from responses with international standard; scale for urines in proportion to the biological activities as determined.

previously described [Harris & Leong, 1936; Harris *et al.* 1938]. Care was taken to effect as complete an adsorption as possible of the vitamin (e.g. by the use of a sufficiently large amount of the acid-clay and attention to various other details of procedure), the effectiveness of the removal being checked by cross tests by the thiochrome reaction. With each specimen of urine, 3 levels of dosage were given corresponding with a calculated intake of 1, 2 and 3 I.U. according to the results of the chemical estimations.¹ Simultaneously, doses of 1, 2 and 3 I.U. of a standard preparation of vitamin B₁ were given, and the responses compared. For the evaluation of results, dose-response curves were constructed (Figs. 2 and 3), and the activity of the urine was calculated in terms of international units (Tables VI and VII).

Since the various specimens of urine were tested biologically in two separate main experiments, carried out at an interval of several weeks apart, separate tests on the standard material were done on each occasion, so as to allow for any variation in the experimental conditions. In series I (Table IV) the bradycardia tests were done by one of us (Y. L. W.) without any previous experience of the method. In the second series (Table V), to reduce any possibility of subjective factors unconsciously influencing the findings, the results of the chemical tests were known only to Y. L. W. and the bradycardia tests were carried out by a laboratory assistant under the direction of L. J. H., neither of the latter having any knowledge of the nature of the different materials under examination. Incidentally, the very close agreement between the series of values "found" and those "calculated", in the last two columns of Table VI, furnishes, we think, further independent evidence of the reliability of the bradycardia method [see also Baker & Wright, 1938; Lunde *et al.* 1938; Pedersen, 1938].

Sensitivity of method

Duplicate tests have been carried out comparing results on the same specimen of urine as well as on other specimens having similar activities. The results indicate that with urines having a fairly high content of vitamin B₁ there is no difficulty in detecting differences in activity of somewhat less than 10%. With urines low in vitamin B₁ differences of between 10 and 20% can be detected. These findings have been confirmed by matchings carried out in this laboratory by workers from three other institutes.

Recovery tests with added vitamin B₁

As a further test of the accuracy of the method, various known amounts of vitamin B₁ were added to different specimens of urine, and the amounts were estimated by the thiochrome test carried out according to the directions given above. Typical examples are given in Table VIII. It will be noted that the percentage recovery varied from 94 to 108, the error being always less than 10% under the conditions studied.

Table VIII. *Recovery of added vitamin B₁*

Urine	Original concentration of vitamin B ₁ μg./ml.	Vitamin B ₁ added μg.	Additional vitamin B ₁ found μg.	Recovery %
(a)	0.04	0.06	0.065	104
	0.04	0.11	0.103	94
	0.04	0.13	0.14	108
(b)	0.085	0.09	0.085	94

¹ Except that with one specimen of urine insufficient material was available for testing more than two levels and with another one level. In these instances the percentage accuracy is somewhat less.

Experiments with test doses

It was previously recommended [Harris *et al.* 1938] that for assessing the level of nutrition of a human subject measurements should be made first of the "resting level" of excretion and then of the response to standard test doses (350 I.U. for an adult of 10-stone body-weight), the latter giving information as to the degree of saturation of the tissues.

The same procedure has been followed in this work with the thiochrome method. Fig. 4 gives examples from two normal subjects and one subnormal

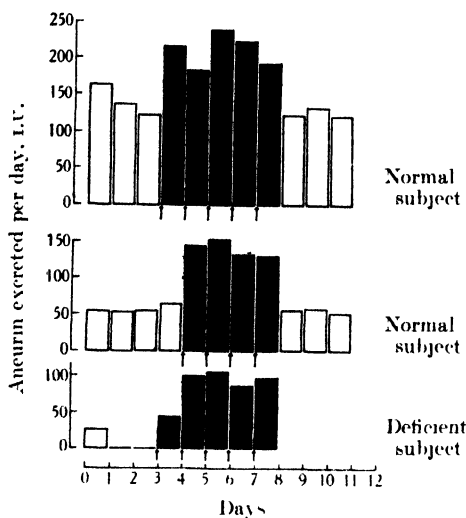


Fig. 4. Resting levels of excretions and response to test doses, in two normal subjects and one deficient subject. Open columns—without test dose. Dark columns—after test dose. Test dose, 350 I.U. per 10 stone of body-weight given at arrow.

case. In the normal individuals an immediate response was seen after the first test dose, the increased excretion remaining approximately constant on each day that a test dose was continued and amounting to about 25% of the extra vitamin given.

With the partially deficient subject the plateau of excretion was not reached until after the second day of test dosing.

On ceasing the dosing, the output of the vitamin almost immediately fell to the original level (Fig. 4).

Standards

In the earlier work by the biological method [Harris *et al.* 1938] the two lowest normal excretions found were 12 I.U. in one series of cases and 15 I.U. in another. In the present more extended series of tests the lowest normal value found was about 30 I.U. We think that the tentative minimum standards previously suggested probably erred in the direction of being unnecessarily conservative. Judging from the present observations, which are based on large numbers of concordant chemical and biological tests, we believe that a value of less than 30 I.U. represents certainly a "sub-average" state of nutrition, and probably it would be fair to describe it as "subnormal". Much more work will be necessary to determine the level of deficiency which may be correlated with the appearance of definite clinical symptoms. A still more difficult task will be to establish a

true "optimum" value, since it is clear that the amount of vitamin B₁ needed for optimal well-being is many times that needed to prevent actual symptoms of disease. However, provision of a simple and reliable chemical test should go far to accelerate progress in this direction. It is just necessary to add that before pronouncing a judgment on any given case it seems advisable to examine the effect of daily test doses given *per os*. A deficient case differs from the normal in showing one or more day's delay before a plateau of excretion is reached [see Harris *et al.* 1938].

As mentioned above, the normal values previously found ranged from about 12 to 40 I.U., as compared with 33 to 130 I.U. in the present tests. It is possible that there was some systematic loss in these earlier preliminary experiments (e.g. during adsorption), but there is no reason to doubt that the original categories were justified in so far as they showed that there was a negligible excretion in beri-beri and nutritional or conditioned polyneuritis, a diminished excretion in the subjects from the poor industrial area as compared with the well-fed controls, and varying degrees of deficiency in medical cases associated with anorexia, restricted diets, faulty absorption or gastro-intestinal obstruction.

SUMMARY

A study has been made of the thiochrome method as applied to urine and the results obtained have been standardized against direct biological tests.

The principal sources of error to be overcome were found to include: (1) incomplete adsorption; (2) interference from (a) non-specific fluorescing substances, (b) other reducing substances, (c) pigments.

With the modifications in the technique now recommended, the accuracy as well as the sensitiveness and simplicity of the method have been improved (a set of 6 or more tests can be carried out within an hour), interference from non-specific fluorescing substances or inhibiting substances reduced and the results obtained have been proved to be accurate when checked against biological assays. Added vitamin B₁ has been recovered with an error of less than 10%. With average urines, differences of less than 10% in activity can be detected, and 5 ml. are sufficient for a determination.

Some special features of the technique recommended are: (1) the omission of any adsorption process, (2) regulation of the amount of potassium ferricyanide added, (3) special treatment of the filter paper and reagents used, (4) removal of interfering substances, both by preliminary washing of the urine with *isobutanol* and subsequent extraction of the *isobutanol*-thiochrome phase, (5) direct visual comparison of fluorescence.

The usual range of excretion for normal subjects was found to be from 50 to 80 I.U. daily, with a maximum of 160 and a minimum of 30 (in place of the more conservative estimate of a minimum of 12-15 I.U. previously tentatively suggested). Test doses should be given to confirm a diagnosis of deficiency; examples are shown.

Lowered reserves of the vitamin were seen in pregnancy and in "conditioned deficiency" attributed to anorexia or faulty utilization.

Acknowledgements. We are indebted to Drs N. Lawrie, W. A. Nicholson, K. Silberstein, H. J. Taylor and J. Yudkin for so kindly placing specimens of urine at our disposal, to Mr A. Ward for careful technical assistance with the experimental animals, and to Mr G. G. Banerji and Dr B. Ahmad for much help in the earlier stages of the investigation.

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CLXVII. A SURVEY OF THE RAT-BRADYCARDIA METHOD OF ESTIMATING VITAMIN B₁

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(Received 1 June 1939)

THE replacement of the first International Standard vitamin B₁ by the crystalline vitamin (Nov. 1938) makes the present time appropriate for a survey of the responses of the vitamin B₁-deficient rat heart to the acid-clay adsorbate and the use of such responses in routine assays.

Using the technique and basal diet recommended by Birch & Harris [1934], work on rat bradycardia was begun in these laboratories in July of that year. Early assays by this method were considered promising and the details of the technique were developed until we were able to maintain a group of 60–80 rats at a suitable degree of deficiency for 12–20 to be available daily for their curative dose. A colony of this number of rats has been studied over the last 3½ years and we have therefore an assembly of responses to the International Standard acid clay sufficient for an indication of the value of the duration of cure of bradycardia as a means of estimating vitamin B₁ activity.

It has been the custom to feed 5 or 6 doses of i.s. acid clay to groups of rats at each of 3 levels, at first once a fortnight, and after the first few months at weekly intervals. During 1935 and 1936 these weekly doses were fed at different groups of levels, e.g. 10, 20, 30 mg., 15, 30, 50 mg. etc., but for the last 2 years the regular doses have been 15, 25 and 40 mg. weekly (apart from short vacations at Christmas and Easter). In Table I will be found a record of the number of responses obtained with each of these latter doses.

Table I. *Number of responses to i.s. acid clay fed at different levels*

Dose level mg.	No. fed weekly	Total at each level
15	6	501
25	6	486
40	6	475

An examination of the 1462 responses (in Table I) yields the following information:

Table II

Dose in mg.	No. of doses fed	Mean response in days	Range in days	Standard deviation σ
15	501	3.10	0–7.0	1.26
25	486	4.49	0–12.0	1.58
40	475	5.97	2–11.0	1.80

Each group of observations is normally distributed about the mean value (Table III).

Table III. *Distribution of the values*

Mean no. of days	σ	$M \pm \sigma$	No. expected between these limits 68.27 %	No. actually obtained	$M \pm \frac{2\sigma}{3}$	No. expected between these limits 50.5 %	No. actually obtained
For 15 mg. 3.10	1.26	1.84-4.36	342	371	2.26-3.94	253	248
For 25 mg. 4.49	1.58	2.91-6.07	332	351	3.44-5.54	245	242
For 40 mg. 5.97	1.80	4.17-7.77	324	303	4.77-7.17	240	225

The agreement between actual and expected numbers is near enough for the distribution to be regarded as normal.

In common with biological responses used for other vitamin assays, the mean values plotted against the logarithm of the doses fall on a straight line (see Fig. 1), and in this respect our findings differ from those of Leong & Harris [1937]. As the graphs show, the responses given by other vitamin-containing substances fall on lines parallel with that obtained from the standard.

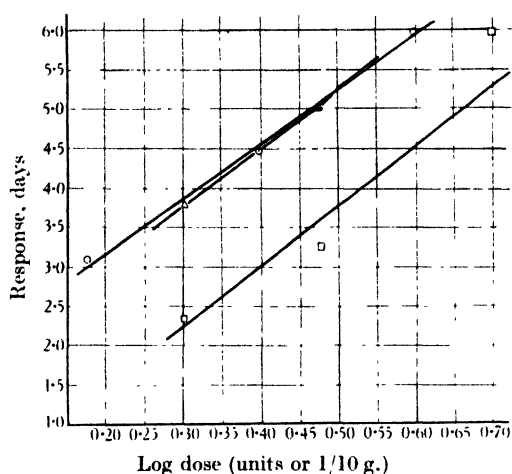


Fig. 1. $y = 6.98x + 1.7466$, \circ — \circ Int. St. acid clay, $y = 7.42x + 1.522$, \triangle — \triangle prepared germ., $y = 7.35x + 0.01$, \square — \square yeast extract.

The variance and the error of the method are obtained as follows:

Table IV. *Variance of responses*

Dose mg.	No.	Mean response (\bar{y})	$\Sigma (y - \bar{y})^2$	$n - 1$
15	501	3.10	792.60	500
25	486	4.49	1216.59	485
40	475	5.97	1542.03	474
			3551.22	1459

$$\text{Variance } (s) = \frac{3551.22}{1459} = 2.434.$$

The equation to the best straight line through these three points is represented by the expression

$$y = 6.980x + 1.7466,$$

where

$$x = \log \text{dose}.$$

It may be shown that the limits of error of a test interpreted with regard to this standard curve are as follows:

Table V. *Limits of error. Acid clay International Standard*

No. of rats	$P=0.95$ %	$P=0.99$ %
40	85-117	83-121
20	80-125	75-133
10	72-138	66-152
5	64-157	55-181

This degree of accuracy can be regarded as satisfactory when compared with that of other biological methods.

Specificity of the method

The method has recently been criticized by Williams & Spies [1938] on the grounds that the response may not be specific for vitamin B₁. In this connexion we have grouped the responses obtained to two natural sources of the vitamin, prepared wheat germ and a yeast extract, samples of both of which we have frequently assayed. The results are given in Table VI.

Table VI. *Cure of bradycardia by natural sources of vitamin B₁*

Source of vitamin	Dose g.	No. of doses	Mean response	Variance	Equation to line relating response to log dose
Prepared wheat germ	0.2	232	3.77	3.73	$y = 7.42x + 1.522$
	0.3	223	5.04		
Yeast extract	0.2	66	2.36	2.91	$y = 7.35x - 0.01$
	0.3	69	3.21		
	0.5	10	6.01		

There is no significant difference between the slopes of these two lines and that of the equation to the i.s. acid clay. This strongly suggests that the active material being measured is the same in each case: any interfering material present in one, would, on this evidence, be equally present in all three.

Precautions taken in the use of the method

(1) During the early months of work on the method, the accuracy obtained was less than it has been more recently. Certain anomalous results, for example, were found to be associated with the use of rats on their first test-dose of a vitamin-containing substance, and it was soon clear that many rats gave a longer response to the first dose than they did to the same size of dose of the same sample given subsequently. When the heart rate first shows the effect of the vitamin deficiency the fact that it falls below 500 beats per minute does not mean that still lower rates can be definitely expected, for, without any dose being given, the rate may be increased again for the next day or two. Some control must thus be exercised in choosing the time for dosage if unreliable results are to be eliminated. Yet if rats with such a fluctuating heart rate are left without any dose until the rate is decreasing steadily, a considerable number die. The practice was therefore adopted of giving each rat an initial dose when the rate was about 450 beats per minute. Cardiographic readings are begun after 28-32 days of vitamin B₁ depletion. The first test dose is deferred until the rate shows a steady regular decline from the maximum recorded after the initial dose.

In most cases in which an abnormally long or a fluctuating response is recorded to the initial dose, the subsequent behaviour of the rat is regular as regards both length and type of response (Table VII).

Table VII. *Typical responses to initial and subsequent doses; beats per minute*

Rat no. 147	
Initial response	433, 500, 460, 480, 455, 480, 400, 488, 450, 420
Response to 1st test dose	420, 450, 460, 412
" 2nd	412, 420, 433, 430, 420, 400
" 3rd	400, 480, 450, 450, 412, 412, 375
" 4th	375, 450, 400, 400, 375
" 5th	375, 450, 350
" 6th	350, 463, 400, 400, 380, 350 (convulsions. Killed by 'HCl ₃)

(2) After a rat has been used for several tests and the rate is under 350 it may die at any time with or without convulsions even soon after eating a dose containing vitamin B₁. For this reason no rat is used if it shows convulsive signs with such a slow rate, and test doses are not given when the rate is below about 330. This practice is regarded as important on account of the behaviour of the control rats, of which there are always two or three.

A rat is allocated as a control when its rate is dropping after its initial dose, the only one it receives. Its life after a reading of about 430 is 6-9 days. It is not unusual for such control rats after showing a lowered heart rate in the expected manner to show an accelerated rate on the day before or the actual day of death. Should such an increase follow a test-dose given to a rat in a severely depleted state, it might be regarded as an indication of vitamin activity and give rise to confused results.

Control rats, 24-hourly readings:

- (1) 433, 410, 400, 386, 360, **375**, 233, death.
- (2) 450, 420, 350, **380**, 300, death.
- (3) 430, 412, 412, 320, **340**, death.

(3) In counting the rate by electrocardiograph tracings, certain abnormalities are occasionally, though not commonly, found in the rhythm. Extra-systoles arise in a few animals; their positions are avoided in the selection of the ends of the length of strip to be counted and they do not affect the record. Rarely a rat shows a form of heart block in which a 24-hourly reading is approximately halved. When this happens it is usually during the early part of its time on test and if the animal is left alone the rate is up again by the following day, e.g.

433 (dose), **290**, 500, 450, 420.
 420 (dose), 525, 470, 440, **200**, 440, 150.
 450 (dose), 488, 500, 480, 470, 450, 460, **300**, 445, 436.

Tracings are also rarely obtained which show dropping of one beat in every three. In 2:1 block the rhythm is counted, not the actual number of beats. These and other changes in the heart of the vitamin B₁-deficient rat have been described by Weiss *et al.* [1938].

(4) Since the rat heart rate responds to such small quantities of vitamin B₁, the absence of all traces of the vitamin from the basal diet is essential. The usual precautions must be taken in preparing the diet, but the behaviour of the control animals remains as a constant check. The presence of traces of the vitamin may prolong the readings sufficiently to produce confusing results.

The bradycardia method has been used for a wide variety of different substances and has been found useful for materials possessing widely different degrees of activity, from a rice containing as little as 0.3 I.U./g. to concentrates

containing 200 or more i.u./g. When the substance to be tested is such that it can be diluted accurately, the method is applicable to materials of high activity such as crystalline aneurin.

Table VIII gives the doses employed for substances of differing activities and the actual limits of error determined directly for each assay.

Table VIII

Sample	Dose g.	No. of doses*		i.u./g.	Limits of error		Limits of potency P=0.95
		Sample	Standard		P=0.95	P=0.99	
Rice A	2.0	20	16	0.9	79-126	74-135	0.7-1.1
	3.0						
Rice B	3.0	20	16	0.3	71-141	64-157	0.2-0.4
	5.0						
Malt food	1.0	20	18	1.0	72-138	67-150	0.7-1.5
	1.5						
Cereal germ A	0.2	19	16	11.4	74-135	68-148	8.4-15.4
	0.3						
Cereal germ B	0.2	20	16	11.5	87-115	82-122	10-13
	0.3						
Cereal germ C	0.2	19	15	12.2	78-128	73-137	9.5-15.6
	0.3						
Yeast extract A	0.2	18	18	11.0	71-141	64-157	7.7-15.4
	0.3						
Yeast extract B	0.3	20	18	8.2	72-139	65-154	5.9-11.4
	0.5						
Yeast concentrate	0.075	12	18	33.1	68-148	60-168	22.4-48.8
	0.150						
Rice concentrate	0.010	10	18	183.2	77-130	71-141	141-236
	0.015						
Germ bread	2.0	10	15	1.2	71-141	64-157	0.85-1.70
	3.0						
Whole-meal bread	3.0	9	18	0.5	60-166	52-194	0.3-0.8
	4.0						

* In each case the dose levels of standard were 1.5, 2.5, and 4.0 i.u.

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OBITUARY NOTICE

EDGARD ZUNZ

1874-1939

THE Society has lost a distinguished foreign member by the death of Prof. Edgard Zunz of Brussels.

Edgard Zunz was born in 1874 in Charleroi. He studied at the University of Brussels and graduated in Medicine with the highest distinction in 1897. He continued his studies in various other laboratories including those of Organic Chemistry at the University of Heidelberg, and of Biological Chemistry at the University of Strasbourg. On his return to Brussels in 1900 he was appointed assistant in the laboratory of Pharmacology and Therapeutics under the direction of Prof. Victor Jacques.

In 1906 he became a full member of the Faculty of Medicine, and from 1909 onwards he undertook the teaching of toxicology to students of hygiene, and that of pharmacography to medical students.

During the war from 1914 to 1918 he did important work as Chief of the Gas Casualty Service and Internal Medicine at La Panne.

In 1919 he was appointed Professor and Director of the Laboratory of Pharmacology and Therapeutics, a post which he continued to hold until his death.

Prof. Zunz became a member of the Royal Academy of Medicine in Belgium in 1919, and served as President in 1934. He was Vice-President of the Permanent Commission of the Belgian Pharmacopoea. He was an honorary graduate of the University of Montpellier, and was a corresponding member of many foreign Academies.

His principal research work was concerned with the digestion of proteins and the properties of protein degradation products; he worked also on the coagulation of blood, on anaphylactic shock, on diabetes and on the secretion of adrenaline. He was further interested at various times in the action of opium alkaloids, in the effects of toxic gases and in the phenomena of adsorption and surface tension.

In addition to his research work he made important contributions to medical literature in writing the chapter on the Coagulation of Blood in the *Traité de Physiologie* of Roger and Binet, and an article on the Regulation and Composition of the Blood in the *Encyclopédie Française*.

His enthusiastic work in a variety of fields brought him a reputation which extended far beyond his own country. His wide scientific knowledge and acquaintance with the literature roused the admiration of all who knew him.

He was greatly interested in art and he found in music the necessary relaxation from his scientific labours.

His death is a severe loss to the University of Brussels and to the numerous medical men and research workers who were formerly students.

CLXVIII. THE SPECIFIC NUTRITIVE REQUIREMENTS OF *CLOSTRIDIUM ACETOBUTYLICUM* (WEIZMANN). II

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(Received 19 June 1939)

IN our first communication [Weizmann & Rosenfeld, 1937] we have shown that *Cl. acetobutylicum* (W.) requires for a normal fermentation of sucrose in a synthetic medium the presence of asparagine and of an unknown compound which has been termed "activator". Thus in accordance with Knight's [1936] classification of micro-organisms by their specific nutritive requirements, *Cl. acetobutylicum* (W.) has a very restricted synthetic power, its nutritional level being comparable with that of numerous pathogenic bacteria.

The unknown part of the nutritive requirements of *Cl. acetobutylicum* hitherto referred to as "activator", has been the subject of further investigation with the ultimate aim of elucidating the chemical constituent or constituents which are responsible for its biological activity. Prior to a further purification and isolation of the "activator" in a chemically pure state it was thought advisable to ascertain whether those chemically defined substances whose growth-promoting properties have already been described in the literature, might also prove to be active in the case of *Cl. acetobutylicum* (W.). In this way it was also hoped to obtain some information about the number of factors involved with the "activator".

I. *The action of compounds of known chemical constitution*

In connexion with the oxido-reduction potential of the medium as a limiting factor for the development of micro-organisms [Kanel, 1937] we investigated the combined influence of cysteine and ascorbic acid. Although we observed a conspicuously beneficial influence on the development of the bacteria in the presence of ascorbic acid, no essential increase of fermentation intensity could be detected. Likewise a negative influence on the fermentation intensity has been found in the case of uracil, nicotinic acid and all possible combinations of this latter substance with aneurin, β -alanine and asparagine.

II. *The action of biotin*

The possibility that a biotin-like substance might be required for the growth and metabolism of *Cl. acetobutylicum* (W.) has already been discussed in our first paper. We are now able to present experimental evidence that biotin, as described by Kögl and co-workers, is one of the essential extraneous factors required by *Cl. acetobutylicum* (W.) for a normal fermentation of carbohydrates to butanol and acetone. The difficulty of preparing chemically pure biotin did not permit a direct test, yet the consistent results obtained by three independent procedures justified the assumption that the experimental responses observed can be ascribed to biotin.

By following the procedure given by Kögl & Toennis [1936] for the extraction and purification of biotin from egg yolk and testing the action of the individual

fractions on the fermentation intensity with *Cl. acetobutylicum* (W.) a clear-cut parallelism was observed between the growth-promoting properties as in the case of yeast found by Kögl, and the stimulation of the butanol-acetone fermentation.

An activator extract from maize which had been subjected to practically the same purification procedure as the above egg yolk extract, showed that the active principle in maize has the same properties as the one from egg yolk, particularly the quantitative adsorbability on charcoal and the eluability therefrom with an ammonia-acetone mixture. For the third independent proof we made use of the different biotin content of seeds which had been quantitatively measured by Kögl & Haagen-Smit [1936]. Parsley and cress seeds being relatively rich in biotin (4900 and 5150 saccharomyces units per g.) have been compared with maize, the latter being relatively poor in biotin. We have found that the ratio of the numbers of activator units of parsley and maize is the same in the case of a normal fermentation with *Cl. acetobutylicum* as that found by Kögl in the case of yeast.

III. The action of the "third factor".

In the experiments where purified egg yolk extract or maize extract were tested for their biotin content and activating power, it was observed that the fermentation velocity failed to increase after the steep initial rise in contradistinction to the corresponding curves obtained with maize or yeast autolysate (see Fig. 1). A similar observation was made both with yeast autolysate treated with acetone and separated from the insoluble part, and with a cress extract rich in biotin. This deviation from the normal behaviour, as in a maize mash, is understood if one assumes that biotin + asparagine account quantitatively only for the initial steep rise of the fermentation velocity curve. The subsequent gradual increase of the curve is caused by a "third factor" which is present in maize or in the dialysate of yeast autolysate, and absent from chemically pure biotin or from purified yeast autolysate.

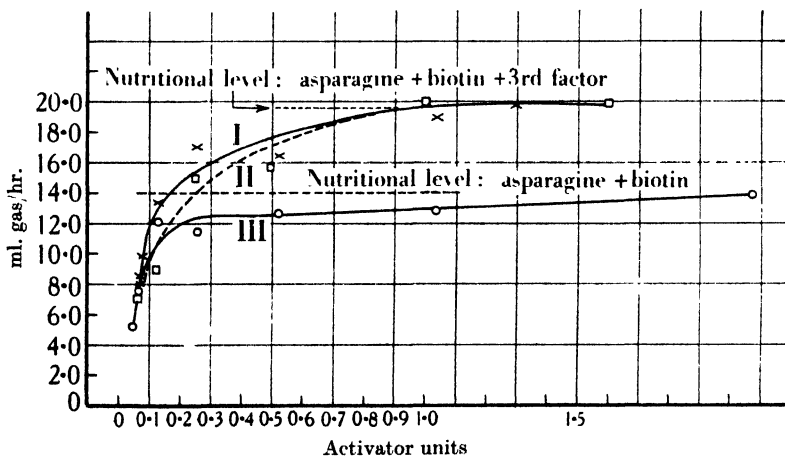


Fig. 1. $t = 36.5$. I (x), dialysate of yeast autolysate; II (□), maize mash; III (○), yeast autolysate, acetone-treated (part soluble in 50% acetone).

An experimental proof of the necessity of a "third factor" in order to obtain a normal fermentation velocity curve as illustrated in Fig. 1, curve I or II,

was given by comparing the fermentation velocity of a system consisting of a solution rich in biotin but poor in the "third factor" with that of a solution devoid of biotin but rich in the "third factor", e.g. a mixture of parsley extract and yeast autolysate (dialysate) which on storage (2 years) had lost its biotin activity. A considerable increase of the fermentation velocity was observed, indicating the existence of the "third factor". This factor is presumably of low molecular weight. It is more stable on storage than biotin and—according to the gradual increase of the fermentation velocity curve which it causes—has a lower biological activity per g. than biotin. For the time being no further assumption is made regarding its chemical nature.

In summarizing the present state of research about the specific nutritive requirements of *Cl. acetobutylicum* (W.) we come to the following conclusion.

If the level of the specific nutritive requirements in maize is taken as 100, then the corresponding level in the presence of asparagine (first factor) amounts roughly to 25%. Owing to the difference in the biotin content of the inoculant used this value shows fluctuations between 4 and 8 ml. gas per hr., the asparagine factor remaining constant.

The second factor, biotin, together with the first factor, asparagine, brings the specific nutritive level up to 70% as compared with maize.

The remaining 30% are due to the "third factor".

EXPERIMENTAL

The general experimental procedure used in this work has already been described in detail in our first paper [1937]. The fermentation intensity as a quantitative measure for the amount of the activating factors has likewise been obtained by graphical evaluation of the fermentation curve and expressed as ml. of gas formed per hr. The fermentation intensity of a 2.5% maize mash served as a standard. In accordance with our earlier experiments the activator content of maize was found to be 1.30 activator units (A.U.) per g. The curve obtained by plotting activator units against fermentation intensity (ml./hr.) from a series of fermentations with varying amounts of maize mash in the presence of 30 mg. asparagine (Fig. 1, curve I) enables the activator content of other solutions or sources to be read, provided that the values for the fermentation intensity are plotted on the same scale.

I. The action of compounds of known chemical constitution

(a) *The influence of ascorbic acid with and without additional cysteine and asparagine.* In a series of 50 ml. round flasks containing 0.60 g. sucrose, 1.0 ml. of 0.80 M phosphate buffer pH 6.8 and 0.15 g. filter pulp, the additions quoted in Table I were made. The sterilized flasks were inoculated with 1 ml. of a culture of *Cl. acetobutylicum* (W.) in 5% maize mash, and incubated.

Table I

$t = 33^\circ$, total volume 38 ml. tap water.

mg. asparagine	30	30	30	30	None	30	None
mg. ascorbic acid	None	2	4	10	10	2	2
mg. cysteine hydrochloride	None	None	None	None	None	15.8	15.8
Fermentation velocity ml./hr.	3.0; 3.6	4.6	4.6	4.4	0.8	4.5	0.6

The experiments with ascorbic acid show a slight increase of the fermentation velocity above the value observed with asparagine alone. The result of the

experiment without asparagine in the presence of cysteine and ascorbic acid (last column) shows that asparagine cannot be replaced by cysteine.

(b) *The influence of uracil.* Uracil has been found to be an essential nutritive factor for the anaerobic growth of *Staphylococcus aureus* [Richardson, 1936]. The fermentation velocity observed in the presence of asparagine and 20 mg. uracil was 2.7 ml./hr. compared with 3.0 ml. for asparagine alone. The experimental conditions were the same as in (a).

(c) *Nicotinic acid, aneurin and β -alanine.* The combinations of nicotinamide or nicotinic acid with aneurin have been proved to be important growth factors for *Staph. aureus* [Knight, 1937]. It was of interest to test these compounds with *Cl. acetobutylicum* (W.). Although negative results with β -alanine have already been reported in our previous communication, the influence of this substance in combination with the others was retested. The amounts per standard experiment in presence of 30 mg. asparagine were:

1.2 mg. nicotinic acid;
2.0 mg. β -alanine;
0.12 mg. aneurin.

All possible combinations have been tried in duplicate. Compared with the fermentation velocity in the presence of asparagine the actions of all compounds and their combinations were invariably negative.

II. *The action of biotin*

The most convenient starting material for the preparation of chemically pure biotin proved to be egg yolk [Kögl & Toennis, 1936]. If biotin were required for the propagation of *Cl. acetobutylicum* (W.) both crude egg yolk and the extracts prepared therefrom should produce an increase of the fermentation velocity in our standard fermentation test. 1.378 kg. egg yolk (51 % dry matter) prepared by boiling 100 eggs for 15 min. and then separating the egg white, served as stock for the subsequent experiments.

A. *Activating effect of egg yolk.* 6.4 g. of moist egg yolk were suspended in tap water and the suspension made up to 100 ml. Varying amounts of this suspension were added to 50 ml. round flasks containing 0.60 g. sucrose, 30 mg. asparagine, 1.0 ml. 0.08 M phosphate pH 6.8 and 0.15 g. filter pulp (standard conditions). The volume was made up with tap water to 38 ml. and the sterilized flasks inoculated as usual. Table II gives the fermentation velocities for the corresponding amounts of egg yolk.

Table II

$t = 34^{\circ}$.

6.4 % egg yolk (mg. suspension moist egg yolk)	None	19.2	38.4	76.8	160	320	640	770 mg. maize as 5 % mash
Fermentation velocity, ml./hr.	5.1	15.6	15.4	15.9	15.3	15.5	15.3	16.0

Although it was to be expected from the figures given by Kögl & Toennis [1936] and Kögl & Haagen-Smit [1936] that egg yolk (3700 s.e./g.) contains about six times as much biotin as maize (600 s.e./g.) which under our experimental conditions would correspond to 130 mg. moist egg yolk, maximum speed was already observed with 19.2 mg. i.e. 1/7 of the calculated amount. It may be that egg yolk contains appreciably more biotin than can be extracted by boiling

water, and that this can be made available from insoluble precursors of biotin by the bacterial enzymes. Further experimental evidence is required for the elucidation of this discrepancy.

1. *Activating effect of egg yolk extract.* 1.35 kg. of the same moist egg yolk tested in (A) were boiled for 1 hr. with approximately 2 l. distilled water and filtered hot. The residue was again heated with 1 l. distilled water and filtered. The joint turbid filtrates (1870 ml.) were treated with acetone (935 ml.), filtered, and the filtrate evaporated *in vacuo* to dryness. The residue was dissolved in 410 ml. water and treated with 1720 ml. 96 % alcohol. After cooling for 1 hr. the solution was filtered and again evaporated *in vacuo*.

The residue was dissolved in 200 ml. water and tested with *Cl. acetobutylicum* (W.) under standard conditions, 1 ml. of the ten-times diluted test solution (= 1) corresponded to 0.68 g. egg yolk. The influence of added inositol was also tested. Table III shows the results.

Table III

ml. acetone- and alcohol-treated egg-yolk extract	0.3	0.3	1.0	1.0	3.0	3.0	10.0	10.0
Corresponding amount of moist egg yolk in mg.	202	202	675	675	2020	2020	6750	6750
mg. inositol	None	11	None	11	None	11	None	11
Fermentation velocity, ml./hr.	9.4	10.0	8.6	11.7	8.9	8.6	10.6	11.1

The maximum speed is already attained with 0.3 ml. of the test solution (0.6 mg. dry matter). Inositol exhibits a small but conspicuous influence, yet it is not able to bring the maximum speed to that of maize mash.

2. *Lead acetate treatment of solution (1).* 193 ml. of solution (1) were treated with 20 ml. saturated lead acetate solution, centrifuged and the clear yellow liquid, which gave no further precipitate upon addition of more lead acetate, treated with H_2S for the removal of Pb. The filtered solution including the washings was evaporated *in vacuo* to dryness. A 0.17 % solution (2) in water was prepared from the dry residue and tested under standard conditions with and without additional inositol. The results resemble, in principle, those given in Table III with the exception that this time inositol was without any influence: average fermentation velocity without inositol, 9.7 ml./hr.; in the presence of inositol 9.8 ml./hr.

3. *Phosphotungstic acid treatment.* To 234 g. of solution (2), 12.5 g. conc. sulphuric acid were added and the mixture was precipitated with 100 ml. 50 % phosphotungstic acid solution in water. After filtration the precipitate was washed with 100 ml. distilled water which had been acidified with 7 ml. 10 % HCl. After decomposition of the precipitate with 2.5 l. 2 % $Ba(OH)_2$, and removal of the excess Ba^{++} with H_2SO_4 , the solution was concentrated *in vacuo* to 197 ml. (solution (3P)). The filtrate, including the washings of the phosphotungstic acid precipitate, was freed from excess of phosphotungstic acid with 150 g. $Ba(OH)_2 \cdot 8H_2O$. Neutralization, removal of Ba^{++} , filtration and concentration gave solution 3S (485 ml.). The activating effects of both solutions 3P and 3S were tested under standard conditions, the results being given in Table IV.

Both the precipitate (3P) and the solution (3S) show activity, yet comparison of the results given in columns 1 and 6 indicates that the precipitate contains at least four times as much of the activating factor as the solution. It

Table IV

 $t = 35^\circ$. 1 ml. 3P = 2.5 ml. 3S.

No. of exp.	1	2	3	4	5	6	7	8	9
ml. solution 3P	0.2	0.5	1.0	1.0	—	—	—	0.5	1.0
ml. solution 3S	—	—	—	—	1.0	2.0	20.0	1.3	2.5
Fermentation velocity, ml./hr.	13.7	15.3	15.5	1.0 No asparagine	9.5	13.4	11.9	10.7	12.4

seems quite possible that the solution 3S owes its activity to the washing with acidified water in which the active compound is partially soluble.

4. *Adsorption on charcoal and elution.* 150 ml. of solution (3P) were shaken for 1 hr. with 15 g. charcoal (Merck, med.) filtered and washed with 200 ml. distilled water; filtrate and washings were evaporated *in vacuo* giving solution 4a, 85 ml. The charcoal was again shaken with 300 ml. 50 % alcohol for 2 hr. The yellow alcoholic filtrate was evaporated to dryness *in vacuo* and dissolved in water, giving solution 4b, 89 ml.

For eluation the charcoal was shaken three times for 2 hr. with 500 ml. of a mixture composed of 220 ml. 17 % NH_3 + 900 g. acetone made up to 1.5 l. with distilled water (60 % acetone, 2.5 % NH_3). The joint eluates were evaporated *in vacuo* to dryness and redissolved in 167.5 ml. water. The solution 4c showed an orange colour. Solutions 4a, 4b and 4c were tested for their activity under standard conditions. Solution 4c (eluate) was diluted ten times for the test. The results are given in Tables V and VI.

Table V

 $t = 34^\circ$.

ml. solution 4c eluate 1	10	0.0	0.2	0.5	1.0	2.0	5.0	10.0	20.0
Fermentation velocity, ml. hr.		4.4	6.7	6.2	5.9	7.4	10.4	10.3	10.4

Table VI

ml. 4a	1.0	1.0	—	—	1.0	1.0
ml. 4b	—	—	1.0	1.0	1.0	1.0
ml. 4c eluate, orig.	—	0.5	—	0.5	—	0.5
Fermentation velocity, ml./hr.	4.4	7.6	9.1	10.4	9.7	10.4

The results in Tables V and VI obtained with *Cl. acetobutylicum* (W.) confirm the results obtained by Kögl & Toennis [1936] with yeast, viz.:

1. The adsorbed biotin is not eluted by water (Table VI, col. 1).
 2. 50 % alcohol removes some of the adsorbed biotin from charcoal (Table VI, col. 3).

3. Biotin is eluted by a mixture containing 60 % acetone and 2.5 % NH_3 .

The solutions obtained from each step of purification show equal responses both when tested for their growth-promoting properties towards yeast and their stimulating influence upon the velocity of a butanol-acetone fermentation of sucrose in a strict synthetic medium. Both effects may therefore be ascribed to the same chemical substance, viz. biotin.

B. *Biotin in maize extract.* If biotin from egg yolk is identical with the activating principle in the standard medium for *Cl. acetobutylicum* (W.), viz. in maize mash it should be possible to obtain analogous results from maize extract using the same purifying procedure as for egg yolk extract.

The maize extract was prepared by stirring 600 g. ground maize with 3 l. N/10 HCl for 4 hr. at room temperature (24°) in the presence of toluene, which procedure, as we knew from previous experiments [Weizmann & Rosenfeld, 1937; Weizmann & Davies, 1937] brings all the activator into solution. The treatment at low temperature has the advantage that the starch can be readily separated afterwards by centrifuging or filtering. The milky filtrate including the washings was exactly neutralized to pH 7.0 with 2N NaOH whereupon the colloidal suspension flocculated out. The solution was again filtered and the filtrate concentrated *in vacuo* to 87.1 g. Analysis showed the N distribution to be as follows:

600 g. maize (1.70 % N) with 10.20 g. N gave $\left\{ \begin{array}{l} 87.1 \text{ g. conc. extract} = 1.15 \text{ g. N} \\ 10.32 \text{ g. precipitate} \\ \text{upon neutralization} = 0.30 \text{ g. N.} \end{array} \right.$

14.2 % of the total N was dissolved during the extraction, 11.2 % is contained in the active concentrate.

For the activator test the concentrated extract was diluted ten times. 1.45 ml. of the diluted extract corresponded to 1.0 g. maize. One series was carried out under standard conditions, in a parallel series the addition of asparagine was omitted. Fig. 2 shows the fermentation velocity observed for a given amount of extract.

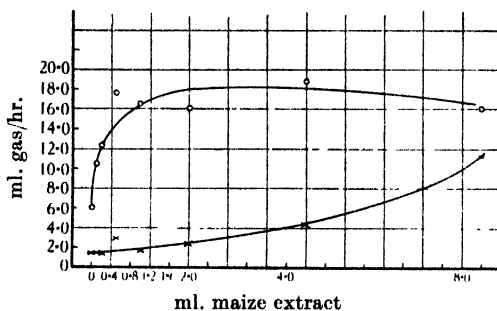


Fig. 2. Maize extract. $t = 35.5^\circ$. o, in the presence of asparagine; x, without asparagine.

The curve without asparagine shows a very low activity indicating the insufficient asparagine content of the test solution. 8 ml. extract (70 mg. protein) are necessary for some asparagine-like action.

Lead acetate treatment of maize extract. To 50 g. extract from the preceding experiment 50 ml. of saturated lead acetate solution were added. After vigorous shaking the precipitate was filtered by suction and the clear yellow filtrate delead with H_2S . The lead-free solution was concentrated *in vacuo* and made up to 48 g. (solution B_2). 5 g. of solution B_2 contained 10.5 mg. N, i.e. 21.6 % of the corresponding amount before lead acetate treatment (48.7 mg. N).

For the test the concentrate was diluted ten times. 1 ml. of the dilute solution corresponded to 0.51 g. maize. Besides the standard test, a parallel series without asparagine was carried out. Table VII shows the results.

Table VII

$t = 35^\circ$.

ml. B_2 (1 : 10)	0.1	0.2	0.5	1.0	2.0	4.0
Fermentation velocity in ml./hr. with asparagine	5.2	9.2	10.9	12.9	17.0	18.5
Without asparagine	0.9	0.6	0.6	0.6	1.2	0.9

As in the case of egg yolk extract there is no removal of the activating factor by the treatment with lead acetate. The inactivity of the "activator" solution in the absence of asparagine demonstrates clearly the dependence of the biotin activity on the presence of asparagine.

Adsorption on charcoal of biotin-containing maize extract. 5g. of solution B₂ (preceding exp.) were shaken with 1 g. charcoal. After centrifuging and washing the charcoal three times on the centrifuge (the last two washings contained colloidal charcoal) the residual (colourless) liquid including the washings was evaporated *in vacuo*, made up to 50 ml., filtered from traces of charcoal and kept for test.

Elution. The charcoal adsorbate was stirred three times with 20 ml. of an acetone-NH₃-water mixture as described in biotin-charcoal adsorbate from egg yolk. A clear yellow eluate was obtained. The combined eluates were evaporated *in vacuo* for the removal of NH₃ and acetone and made up to 50 ml. The test was carried out as usual. The results are given in Table VIII. 1 ml. eluate corresponds to 0.51 g. maize.

Table VIII

$t = 35.0^\circ$.

ml. eluate	0.0	0.5	1.0	2.0	5.0	10.0	15.0	0.0	5.0
ml. washing	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0	5.0
Fermentation velocity, ml./hr.	4.2	8.9	9.6	12.8	11.4	13.1	13.0	4.2	11.3

The eluate is active but the maximum speed obtained is only 70% of that observed in the preceding purification steps, where the maximum speed was equal to that of a maize mash. Apparently a factor required for a normal fermentation velocity has been eliminated in the last purification (third factor). In case this factor has not been adsorbed by charcoal it should be possible to reconstruct the activating system as it was before adsorption by combining the eluate with the washings. Yet the experimental test of such a combination showed a negative response, as may be seen from the last column in Table VIII. One is therefore led to assume that the eliminated factor has also been adsorbed like biotin on charcoal, but could not be eluted with acetone-NH₃ mixture. Further experimental evidence on the adsorption of the "third factor" on charcoal is given in the third section of the experimental part. No difference in the behaviour of biotin from egg yolk and from maize has been observed.

C. *The ratio of biotin contents in maize, parsley or cress seeds.* For the preparation of the seed extracts care was taken that the conditions for the extraction were identical in all three cases. The extraction was carried out at 40° with N/100 HCl. In every case during the extraction the ratio of HCl to seed was chosen so as to keep the acidity at pH 5. This procedure differs from that recommended by Kögl & Haagen-Smit [1936], viz. heating the ground seed with water for 30 min. at 100° and separating the biotin solution from the seeds by ultra-filtration. Firstly, owing to evaporation during ultra-filtration by suction it has seemed to us difficult to obtain a reliable aliquot part from a given amount of liquid; secondly, the treatment of maize at 100° causes swelling of the starch which cannot then be separated by simple centrifuging or filtration; finally, as was already pointed out, the recovery of biotin by our procedure is quantitative.

Maize. 200 g. ground maize were stirred with 1000 ml. N/100 HCl for 3½ hr. at 40°. The pH during the extraction was 4.7. An aliquot part of the centrifuged extract was neutralized to pH 7.0 with N NaOH. After filtration from the

flocculent precipitate an aliquot part of the filtrate was concentrated *in vacuo* to 323 g. which corresponded to 117.5 g. maize. (1 g. maize = 2.75 ml. extract.) The activity of the extract was tested in the customary manner. Table IX gives the corresponding results.

Table IX

$t = 36.5^\circ$.				
ml. extract	0.0	0.2	0.4	0.8
Fermentation velocity, ml./hr.	5.1	13.0	15.7	18.3
Activator units	(50 mg. maize)	(123 mg. maize)	(200 mg. maize)	(350 mg. maize)
	0.065	0.16	0.26	0.40

Parsley. 87 g. milled parsley (light petroleum-extracted) were stirred with 1000 ml. $N/100$ HCl for $3\frac{1}{2}$ hr. at 40° , pH during extraction 5.2. An aliquot part of the centrifuged extract was neutralized with NaOH to pH 7.0. After filtration from the precipitate formed, an aliquot part of the filtrate was concentrated *in vacuo* to 240 g. which corresponded to 50.8 g. parsley (defatted). 1 g. parsley = 4.72 ml. extract.

Cress seeds. 52 g. milled and defatted garden cress seeds were stirred for $3\frac{1}{2}$ hr. at 40° with 780 ml. $N/100$ HCl. The pH during the extraction was 4.9. The separation of the extract from the insoluble residue was rendered difficult by the swelling of constituents of this type of seed. After neutralization, filtration and evaporation 134 g. concentrated extract were obtained which corresponded to 21.05 g. defatted seeds. For the activity test the solution was diluted ten times.

The results of the activity test together with those obtained from the parsley extract are given in Table X.

Table X

ml. parsley extract	0.1 21 mg. parsley	0.2 42 mg. parsley	None	None	None	None
ml. cress extract	None	None	None	0.2 12.7 mg. cress	0.4 25.4 mg. cress	0.8 51 mg. cress
Fermentation velocity, ml./hr.	14.6	20.4	5.7	12.3	13.7	12.8

Evaluation of the results obtained with maize, parsley and cress extracts

The speed values obtained from the fermentation experiments with our reference media, viz. maize extract (in the presence of asparagine) are plotted in

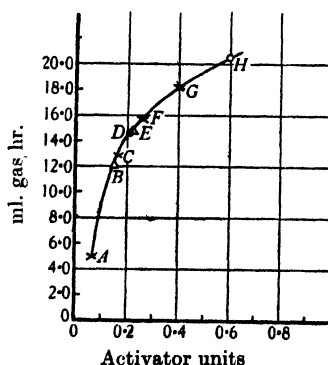


Fig. 3. A = 50 mg. maize; B = 12.7 mg. cress; C = 123 mg. maize; D = 21 mg. parsley; E = 25.4 mg. cress; F = 200 mg. maize; G = 350 mg. maize; H = 42 mg. parsley.

Fig. 3 as activator units (A.U.) against fermentation velocity (ml./hr.). Within the limit of experimental error the speed values observed with maize extract are the same as those obtained with the corresponding amount of maize mash under the same conditions. The activator content of the HCl extract deriving from 1 g. maize was therefore taken as 1.30 A.U. The speed values obtained from the fermentations with parsley and cress extracts were plotted on the curve obtained from maize extract. The corresponding activator contents were read on the abscissae.

The A.U. values thus obtained from Fig. 3 are:

Maize extract	Parsley extract	Cress extract
Corresponding to 123 mg. maize	42 mg. parsley = 0.60 A.U.	12.7 mg. cress = 0.15 A.U.
= 0.16 A.U.	21 mg. parsley = 0.21 A.U.	25.4 mg. cress = 0.23 A.U.
i.e. 1.3 A.U. per g. maize	i.e. mean 12.2 A.U. per g. parsley (defatted)	i.e. mean 10.5 A.U. per g. cress seed (defatted)
	9.8 A.U. per g. parsley (orig.)	8.1 A.U. per g. cress seed (orig.)

For comparison of our results with the biotin contents of maize, parsley and cress as obtained by Kögl & Haagen-Smit [1936] we transform Kögl's saccharomyces units into the corresponding activator units. Accordingly maize contains 600 saccharomyces units (S.E.) or 1.3 activator units (A.U.) per g. parsley contains 4900 S.E. or 10.6 A.U. per g. and cress contains 5150 S.E. or 11.2 A.U. per g. Despite the difference in the test methods used the agreement of our values with those of Kögl is satisfactory.

III. Experimental evidence for the existence of the "third factor"

We tried to find a convenient laboratory method for the preparation of activator from yeast autolysate which avoids both the difficult separation of the active solution from the colloidal constituents of the yeast cell, or the tedious process of collection and evaporation of the dialysates of yeast autolysate. We overcame these difficulties by treating the yeast autolysate with an equal volume of acetone and separating the active solution from the insoluble parts by centrifuging. By evaporating the acetone solution *in vacuo* a dry orange coloured hygroscopic product was obtained. Table XI shows the results of the activity test with this product.

Table XI

mg. dry autolysate	0.0	1.45	2.9	5.8	11.5	23.0	46.0
Fermentation velocity, ml./hr.	5.2	7.6	12.1	11.5	12.7	12.9	13.9
A.U.	0.050	0.065	0.13	—	—	1.04	2.07
Without added asparagine							
mg. dry autolysate	92.5	92.5	108	145	250	500	
Fermentation velocity, ml./hr.	17.9	20.1	18.4	22.0	26.0	27.6	
A.U.	4	4	—	—	10	20	

In the presence of 92.5 mg. dry autolysate the addition of asparagine has no stimulating effect upon the fermentation velocity, the optimum amount is already contained in the amount of autolysate used. Therefore an addition of asparagine at higher amounts of autolysate has been omitted.

The fermentation velocities have been plotted as curve III in Fig. 1. While the corresponding test with the dialysate of yeast autolysate (prepared according to the procedure given in our previous paper), curve II, Fig. 1 gives a fermentation velocity curve practically identical with the one obtained with maize mash

(curve I, Fig. 1), the acetone-treated yeast autolysate (curve III) follows the other curves only in the initial part up to 0.13 A.U. After that point the fermentation velocity remains fairly constant to 2 A.U. Up to this point both the yield of solvents and the titration acidity remain normal. According to the activating properties the autolysate obtained by separating the insoluble matter (yeast cells together with some protein) with acetone is different from those autolysates which have been obtained either by filtration or by dialysis. The acetone treatment removed a factor (we term it "third factor") from the autolysate which in maize mash, yeast autolysate or dialysate of yeast autolysate causes a further increase of the fermentation velocity curve (I and II, Fig. 1) from 0.13 to 1.0 A.U. A deficiency of the same character, viz. the failure to attain maximum speed observed in the case of a maize mash, was also met with in the charcoal eluates of biotin from purified egg yolk and maize extracts (Tables V and VIII) and in cress extract (Table X).

The increase of the fermentation velocity above 2 A.U. (Table XI) has a different biochemical significance, the yields of alcohol and acetone decreasing, and the titration acidity increasing. At 20 A.U. practically no butanol and acetone are formed, the fermentation products being only butyric and acetic acids. The same observation was made also with parsley mash. At first sight it seemed that overdoses of biotin change the butanol-acetone fermentation into a butyric acid fermentation. Yet one has to take into consideration that the buffering capacity of the media is also increased by the addition of large amounts of yeast autolysate or parsley mash. (Parsley mash required for the same change of pH three times as much acid as maize mash.) In this case the media would acquire the properties of maize mash plus CaCO_3 . A final answer concerning the biochemical significance of this interesting observation must be postponed until the experiment has been repeated with chemically pure biotin.

An experimental proof of the existence of the "third factor" was given by combining a solution rich in biotin but poor in the "third factor" with a solution poor in biotin but rich in the "third factor". Parsley extract is an example of the "biotin" solution. As a solution of the "third factor" we used a dialysate of yeast autolysate which had entirely lost its biotin activity after 2 years' storage (dialysates D_1 and D_2 in our earlier paper). The results of the subsequent experiments are mean values from three parallel runs; the mean deviation from the stated fermentation velocity is also given.

The charcoal treatment with D_1 and D_2 (15 ml. D_1 or D_2 + 3 g. Merck's charcoal were shaken for 5 min. and filtered) was carried out in order to remove traces of biotin eventually present in the stored dialysates.

A. Solutions rich in biotin

	Fermentation velocity ml./hr.
Parsley extract { 0.1 ml.	11.9 \pm 0.41
0.5 ml.	13.4 \pm 1.16
Yeast autolysate 1.7 ml.	13.5 \pm 0.46

B. Solutions containing the "third factor"

	Fermentation velocity ml./hr.
Dialysate of yeast autolysate D_1 (1936) { 0.1 ml.	6.03 \pm 1.73
0.2 ml.	7.07 \pm 0.18
Dialysate of yeast autolysate D_2 (1936) { 0.6 ml.	2.7 \pm 0.22
1.2 ml.	2.1 \pm 0.0

Combinations of A and B

		Fermentation velocity ml./hr.			Fermentation velocity ml./hr.
1. Parsley extract	{0.1 ml. }	16.7 \pm 0.76	Parsley extract	{0.5 ml. }	16.2 \pm 0.6
D ₁	{0.12 ml. }		D ₁	{0.12 ml. }	
2. Parsley extract	{0.1 ml. }	13.9 \pm 0.28	Parsley extract	{0.5 ml. }	18.2 \pm 0.42
D ₁ (charcoal-treated)	{0.13 ml. }		D ₁ (charcoal-treated)	{0.13 ml. }	
3. Yeast autolysate	{1.7 ml. }	17.4 \pm 1.9			
D ₂	{0.6 ml. }				
4. Parsley extract	{0.1 ml. }	12.1 \pm 0.49	Parsley extract	{0.5 ml. }	14.0 \pm 0.0
D ₂ (charcoal-treated)	{0.75 ml. }		D ₂ (charcoal-treated)	{0.75 ml. }	

Results

Combination 1 (untreated D₁) shows a positive activation of the biotin solution in the form of parsley extract.

Combination 2 (D₁ charcoal-treated) shows a positive but weak activation of the biotin effect obtained by parsley extract alone. With more parsley extract full activation was obtained.

Combination 3 (D₂ as source of the "third factor" with a freshly prepared dialysate as a source of biotin) shows together a positive activation.

Combination 4 (D₂ charcoal-treated with parsley extract as a source for biotin) showed no activation; in distinction to Combination 2 an increased amount of parsley extract gave likewise a negative response. The "third factor" in dialysate D₁ seems to be present in a higher concentration than in dialysate D₂. The "third factor" is to some extent adsorbed by charcoal; this has already been indicated by the results quoted in Table VIII.

The presence of the "third factor" in D₁, i.e. the first fractions obtained by dialysis of yeast autolysate, points to the fact that the factor in question is substance of low mol. wt.

DISCUSSION

The results of our experiments have revealed that one of the hitherto unknown nutritive factors which are required by *Cl. acetobutylicum* (W.) for a normal butanol-acetone fermentation is identical with biotin, a substance which, according to Kögl, promotes the growth of yeast as one of the bios factors. These two actions of biotin, viz. the stimulation of yeast growth and the stimulation of the fermentation intensity of a butanol-acetone fermentation can be correlated with one another by the intelligible assumption that the increased fermentation velocity (ml. gas/hr.) due to the presence of biotin is proportional to the concentration of the bacterial metabolic enzymes which in turn are proportional to the bacterial population. Biotin may, therefore, also promote the growth of *Cl. acetobutylicum* (W.). This assumption still requires direct experimental confirmation, yet it has acquired a high degree of probability since Kögl & Fries [1937] and Kögl & v. Wagtendonk [1938] have shown that the growth-promoting action of biotin is not restricted to a particular strain of yeast, but is also required by some fungi and by *Staph. aureus* in addition to aneurin and nicotinamide [Knight, 1937]. It is quite possible that biotin might prove to be an active growth stimulant with other bacteria, where it has not yet been suspected, e.g. with *Bact. anthracis* [Landy, 1939].

The main reason why we did not confine our measuring procedure to the estimation of the influence of the medium upon the growth of the bacterium was based on the following fact. The media which stimulate the growth of *Cl. acetobutylicum* (W.) do not necessarily produce a fermentation in which the yields

of alcohol and acetone, the final titration acidity and the fermentation velocity are identical with the butanol-acetone fermentation of a maize mash, to which we always refer as a normal fermentation. Maize contains all the specific nutritive requirements for *Cl. acetobutylicum* (W.) in a particular harmonic combination. We endeavoured to look at the fermentation as a whole; we did not accept, for example, any fermentation velocity as a true measure for the activator content unless the yields of products and the titration acidity were normal. In many instances we tested microscopically the appearance of clostridia and spores as a sign that the bacteria had completed their normal life cycle.

For the comparison of experiments which had been carried out at temperatures different from 37°, or at a different total volume, use was made both of the temperature coefficient of the fermentation velocity which, between 33 and 38°, was found to be 2_{10° and of a simple relationship which exists between the fermentation velocity and the volume of the medium, viz. fermentation velocity/volume = K. In the case of a normal medium e.g. maize mash K_{37° was found empirically equal to 0.6. For example 40 ml. of a 2.5% maize mash showed a fermentation velocity of 24.0 ml./hr. at 37° and 38 ml. of a 2.5% maize mash showed a fermentation velocity of 16.0 ml./hr. at 34°. The calculated value was 15.95 ml./hr.

The above-mentioned normal value for K, viz. 0.6 was found to be much higher with a 2.5% parsley-mash-sugar medium, viz. $K_{37^\circ} = 1.2$ (35.0 ml. gas/hr., 34°, total volume 38 ml.). Yet in this particular case the fermentation cannot be called a normal one as no alcohol or acetone has been formed but only butyric and acetic acid. Despite the complexity of the activating system of *Cl. acetobutylicum* (W.) the activator unit (A.U.) as defined in our previous paper has been adhered to for practical reasons. Owing to the "third factor" the deviations from the normal shape of the fermentation velocity curve above 0.15 A.U. had to be taken into account. In case of a deficiency in the "third factor" the activator content should be measured in the range of 0.05–0.15 A.U. of the fermentation velocity curve.

From curve III, Fig. 1, which described the influence of biotin in the presence of asparagine (as far as such a curve can be abstracted from experiments if only a small amount of the "third factor" was present) it can be seen that a further increase of the biotin concentration above 0.15 A.U. has no essential influence upon the fermentation velocity. 0.15 A.U. seems to be the optimum amount of biotin required by the bacteria under the given experimental conditions. The multiplication of the bacteria at this stage amounts to 4000% (unpublished experiments); the approximate amount of A.U. corresponding to a 100% multiplication, as in Kögl's test, would be of the order of $0.15 \times 1/40 = 0.0038$ A.U., and as $1.0 \text{ A.U.} = 540 \text{ saccharomyces units (Kögl)}$, $0.0038 \text{ A.U.} = 2.0 \text{ s.e./40 ml.}$ and therefore $0.0038 \text{ A.U.} = 1.3 \text{ s.e./25 ml.}$

This calculation is naturally only approximate, as the multiplication of the bacteria is not strictly proportional to the biotin concentration (Kögl). The figures obtained have therefore only the qualitative significance that the biological action of one saccharomyces unit ($2.5 \times 10^{-4} \mu\text{g.}$ biotin) is probably of the same order both in the case of *Saccharomyces cerevisiae* and *Cl. acetobutylicum* (Weizmann).

SUMMARY

1. *Clostridium acetobutylicum* (Weizmann) belongs to those heterotrophic bacteria whose synthetic power is very restricted; it cannot synthesize the factors required for growth.

2. Even in the presence of asparagine, combinations of aneurin, nicotinic acid and β -alanine, or uracil cannot replace the growth factors present in maize or yeast.

3. It has been demonstrated that one of the active growth factors required for *Cl. acetobutylicum* (Weizmann) is biotin.

4. In order to bring the nutritive level of a synthetic medium up to 70 % of the nutritional level represented by a maize mash both asparagine and biotin must be present.

5. The missing 30 % of the full nutritional requirements is due to a "third factor", for the existence of which experimental evidence has been brought forward.

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CLXIX. SEASONAL VARIATIONS IN THE VITAMIN A CONTENT OF CERTAIN VISCERAL ORGANS OF THE GEELBEK OR CAPE SALMON (*TRACTOSCION AEQUIDENS* C. AND V.)

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DURING a general survey of South African fish oils which is now in progress, it became probable that the considerable variations in the vitamin A content of certain oils were in some way associated with the intensity of feeding of the fish. As it was feasible that such variations might throw some light on the role of vitamin A in fish, a seasonal study was made, over periods of intensive feeding and of starvation, of both the liver and the visceral oils of the geelbek or Cape salmon. By visceral oils are meant here the combined oils from the pyloric caeca and the intestines.

EXPERIMENTAL

Sampling. Rather a small number of fish (3–15 according to their availability) have been taken for each sample. Under ordinary conditions the sampling error with so few fish would be considerable, but individual variations have been reduced to a minimum by selecting whenever possible only female fish weighing between 12 and 15 lb. In addition all fish were rejected which did not conform to the general trend with regard to the size of the liver. The need for obtaining uniformity in regard to the size of the liver is not so apparent in November and December, when all the fish are in poor condition, with a small shrunken liver and a low oil content. But later, when they commence feeding actively, the rate of increase in the size of the liver varies tremendously, with the result that it is possible to obtain fish of the same size, the weights of whose livers are in a ratio of more than 5 to 1.

Methods of extraction. With certain samples of high oil content, oil for analysis was obtained by steaming. Where the oil content was low, however, the tissues were minced and desiccated with anhydrous sodium sulphate and the oil was then extracted with light petroleum (B.P. 30–50°) or peroxide-free ether. In all cases, the oil content of the tissues was estimated by this latter method.

Analytical procedures. Iodine values were determined by the Wijs method. Vitamin A was estimated by determining $E_{1\%}^{1\text{cm}}$ at wave-length 325–330 m μ , and using the conversion factor 1600 for expressing it as a percentage of vitamin A [Carr & Jewell, 1933]. A Hilger quartz E. 3 spectrograph was used in conjunction with a sector photometer. The solvent was either absolute alcohol or cyclohexane.

The results of the investigation are recorded graphically. For the construction of these graphs 2–4 samples were collected and analysed each month. In order to eliminate individual variations in the samples as far as possible, the results have been averaged over each month.

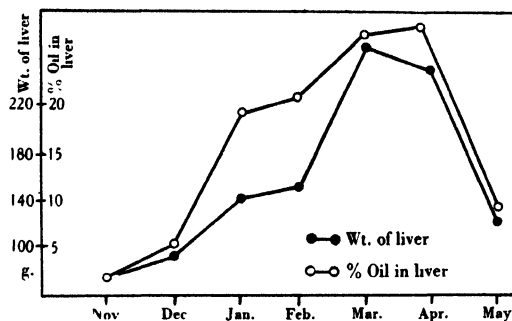


Fig. 1. Seasonal variations in the weight and oil content of the liver of the geelbek or Cape salmon.

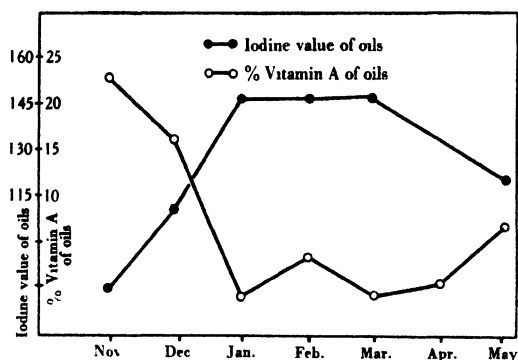


Fig. 2. Showing the inverse relation between the iodine values of geelbek liver oils and their vitamin A content.

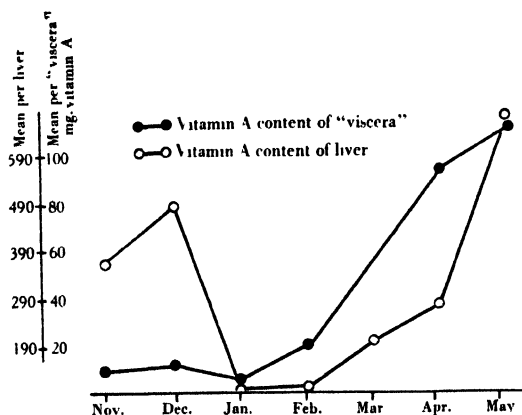


Fig. 3. Seasonal variations in the total vitamin A content of the liver and of the "viscera" of the geelbek.

From Figs. 1 and 2, it will be seen that the geelbek which are caught in November are characterized by having small livers with a very low oil content. This oil has an exceedingly high vitamin A content and a very low iodine value. As the season advances and the period of intensive feeding begins, there is a

marked rise in the iodine value of the oil and a decrease in the concentration of vitamin A, while at the same time the liver increases in size and in yield of oil. In Fig. 3, however, it is seen that the total vitamin A per liver decreases over this period to a mere fraction of its former value, so that the reduced percentage of vitamin A is not due entirely to infiltration of fat. At the same time, the visceral vitamin A per fish increases rapidly from the time the fish starts feeding intensively. During the autumn months there is a gradual increase in the amount of vitamin A in the liver, while at the same time the iodine value of the oil begins to fall and the liver decreases in size. By June the geelbek have more or less completely disappeared from False Bay (where all the samples were obtained), or at least are no longer to be caught by the fishing boats. When the fish reappear in October and November they are in poor condition and have obviously been through a period of starvation. The total vitamin A per liver has, however, been affected to only a minor extent.

DISCUSSION

It is clear that in the geelbek, a decrease in the total vitamin A content of the liver accompanies increased intensity of feeding. So far, there have been few, and these incidental, observations made on the seasonal variations in the vitamin content of the liver itself. Most workers have confined themselves to studying the changes in the vitamin A content of the oil, and not in the total vitamin A of the liver. Bills *et al.* [1934] have noted that the potency of halibut liver oils is related to the oil content of the livers, and have stated that the decrease in the vitamin A content of the oil from January to August is far too great to be accounted for by the increased oil content of the liver. Confirmation of this view is obtained if the results reported by Haines & Drummond [1936] and by Evers *et al.* [1936] with certain halibut liver oils are examined. If the periods of increasing oil content of the liver and of increasing iodine value of the glycerides are taken as the periods of increased intensity of feeding, then these results are in harmony with our own recorded here. The studies of Shorland [1938] on the seasonal variations in the vitamin A content of ling liver oils, and the work of Bailey [1933] on the ling cod liver oils can also be interpreted as indicating the effect of increased intensity of feeding in tending to deplete the vitamin A reserves of the liver.

These observations are of particular significance in view of the suggestions [Edisbury *et al.* 1938; Lovern *et al.* 1939; Lovern & Morton, 1939] that vitamin A may play some role in the process of fat assimilation in certain fish. The diet of the geelbek during the period of intensive feeding is a very fatty one, consisting of sardines (*Sardinia* spp.), maasbankers (*Trachurus trachurus* Linn.) and Cape mackerel (*Scomber colias* Gmal.). An analysis of two sardines caught in November showed them to contain about 5 % of fat (blue value in 20 % solution = about 1), while Juritz [1922; 1933] has reported the oil contents of samples of maasbankers and Cape mackerel examined by him as 6.9 and 11.8 % respectively. The question therefore arises as to whether the fall in the total vitamin A content of the liver at the commencement of intensive feeding is in any way related to the fact that the fish is at this time increasing its fat reserves.

So far there has been no work published on seasonal variations in the vitamin A content of the pyloric caeca and the intestines of fish. The present studies indicate that the amounts of vitamin in these organs are lowest when the fish is in poor condition and highest when it is fat. In other words, the vitamin A content of the viscera rises as their fat content rises. The exact significance of

this is difficult to decide, but it certainly accords with the suggestion that the vitamin may be involved in the transport of fat from these organs.

The effect of starvation in reducing the iodine values of the body and other oils of fish is well known, though in the case of certain liver oils with their high content of sometimes very unsaturated unsaponifiable matter, this effect is not as marked as with body oils. In the case of the geelbek, however, the effect of starvation on the iodine values of the liver oils is very marked. In fact, in some cases the iodine value can be almost all accounted for by the vitamin A of the unsaponifiable matter, indicating the presence of highly saturated glycerides and the more or less complete absence of unsaponifiable matter of high iodine value apart from the vitamin A. In geelbek liver oils the iodine values vary inversely with the vitamin content. These results are in contrast with those available for the New Zealand groper [Shorland, 1937] and the halibut [Evers *et al.* 1936; Haines & Drummond, 1936]. In both these cases the iodine values of the oils and their vitamin A contents show a direct relationship.

SUMMARY

In the geelbek or Cape salmon the total vitamin A of the liver decreases at the onset of intensive feeding and afterwards gradually increases.

The vitamin A content of the visceral oils is directly related to the oil content of the viscera, being greatest when the fish is fat.

There is a direct relationship between the amount of oil in the liver and its iodine value, this latter varying inversely with the percentage of vitamin A in the oil.

These results are in accord with the view that vitamin A is associated with the process of fat assimilation in the geelbek.

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CLXX. VITAMIN B₁ IN THE ANIMAL ORGANISM

III. THE MAXIMUM STORAGE OF VITAMIN B₁ IN VARIOUS SPECIES

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(Received 15 July 1939)

SEVERAL studies have been made of the influence of diet on the vitamin B₁ content of the tissues of the rat [e.g. Osborne & Mendel, 1923; Westenbrink, 1932; Brodie & MacLeod, 1935]. Leong [1937, 1] found that "saturation" of the tissues of the rat with vitamin B₁ occurred when the intake was about 30 I.U. daily and that no further increase of reserves could be found when the intake was raised above this level. The two main sites of storage were the liver and the skeletal muscle and the maximum concentrations of the vitamin in these two tissues were 2.6 and 0.6 I.U. per g. respectively. Similar studies have recently been reported by Schultz *et al.* [1939], whose findings were in general agreement with those obtained by Leong [1937, 1].

As such quantitative studies may contribute to our understanding of the function of this vitamin in the animal body, further investigations into the extent of its storage in other species (*viz.* guinea-pig, fowl and pigeon) were carried out. It appears established that below a certain level of intake (*i.e.* the level that will result in maximum storage) the concentration and also the amount that can be stored are directly proportional to the amount ingested [Leong, 1937, 1; Schultz *et al.* 1939]. It was therefore decided to determine only the levels of maximum storage in these experimental animals. The results will also be of interest as they will show whether or not the levels of maximum storage in the different species are of a similar magnitude.

EXPERIMENTAL

Adult animals were employed and they were kept on their respective diets (which were supplemented with large amounts of vitamin B₁) for a period of about 3-4 weeks. After this they were killed by decapitation and their tissues assayed for vitamin B₁. The following is a description of the diets.

Guinea-pig. The diet was made up of oats, bran and arachis oil, in which mixture was incorporated 20% of an activated acid clay. The latter contained about 125 I.U. of vitamin B₁ and was supplied through the courtesy of Dr A. Z. Baker. Since about 30 g. of food were consumed by each of the guinea-pigs per day, their daily intake was about 750 I.U. vitamin B₁, *i.e.* several hundred times the daily requirement. This diet was also supplemented with green cabbage every day.

Fowl and pigeon. The diet consisted mainly of oats and whole wheat. Each bird received by mouth about 250 I.U. of vitamin B₁ daily in the form of a watery suspension of 2 g. of the activated clay.

Method of assay. The bradycardia method [Drury & Harris, 1930; Birch & Harris, 1934] was employed for assaying the tissues. Comparative tests with

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graded doses of the International Standard were also carried out simultaneously. The responses produced by a given dose of the International Standard acid clay were of the same average magnitude as those obtained on previous occasions. The vitamin B₁ content of the substance under test was read off from a dose-response curve already published by Leong & Harris [1937]. Since it has been found [Leong, 1937, 1] that the combined reserves of the vitamin in the liver and skeletal muscle represent 80–90 % of the total amount stored in a "saturated" rat, only these two tissues were assayed.

RESULTS

The results are given in Tables I and II. For the purpose of comparison the results obtained for "saturated" tissues of rats [Leong, 1937, 1] have also been

Table I. *Maximum storage of vitamin B₁ in the muscle of the following species: guinea-pig, fowl, pigeon and rat*

Species	Animal no.	Days on diet	Cure of bradycardia days*	Average period of cure days	I.U. vitamin B ₁ per g.
Guinea-pig	1	23	1, 1	1.0	0.2
	2	26	2, 3	2.5	0.5
	3	30	1, 2	1.5	0.3
				Av. for guinea-pig	0.3
Fowl	1	20	1, 1	1.0	0.2
	2	25	2, 2	2.0	0.4
	3	29	1, 2	1.5	0.3
				Av. for fowl	0.3
Pigeon	1	21	4.5, 5.5	5.0	1.2
	2	24	4, 5	4.5	1.0
	3	26	4, 5	4.5	1.0
	4	30	6, 6	6.0	1.5
				Av. for pigeon	1.2
Rat [Leong, 1937, 1]				Av. for rat	0.6

* Each figure represents the result of one test.

Table II. *Maximum storage of vitamin B₁ in the liver of the following species: guinea-pig, fowl, pigeon and rat*

Species	Animal no.	Days on diet	Cure of bradycardia days*	Average period of cure days	I.U. vitamin B ₁ per g.
Guinea-pig	1	23	1, 2	1.5	0.6
	2	26	1.5, 1.5	1.5	0.6
	3	30	2, 2	2.0	0.8
				Av. for guinea-pig	0.7
Fowl	1	20	2, 2	2.0	0.8
	2	25	1, 2	1.5	0.6
	3	29	1, 2	1.5	0.6
				Av. for fowl	0.7
Pigeon	1	21	2, 2.5	2.3	0.9
	2	24	2.5, 3	2.8	1.1
	3	26	2, 3	2.5	1.0
	4	30	3, 3.5	3.3	1.4
				Av. for pigeon	1.1
Rat [Leong, 1937, 1]				Av. for rat	2.6

* Each figure represents the result of one test.

included in these tables. It will be seen that the maximum level of storage of the vitamin in the skeletal muscle was quite different for each species. Thus (see Table I), the maximum concentration in the muscle of the guinea-pig and the fowl was of the order of 0.3 I.U. per g., as compared with the value of 1.2 I.U. per g. for pigeon muscle and 0.6 I.U. per g. for rat muscle. The vitamin was present in the livers of the four species (viz. guinea-pig, fowl, pigeon and rat) in the following concentrations: 0.7, 0.7, 1.1 and 2.6 I.U. per g. respectively (see Table II).

In view of the high vitamin B₁ intake and the finding that "saturation" of rats' tissues was attained in a few days after rats were fed on diets rich in this vitamin [Leong, 1937, 2], it seemed reasonable to presume that the tissues of the animals used in this investigation were "saturated" with the vitamin. The correctness of this assumption is supported by the finding that there was no appreciable difference in the concentrations of the vitamin in, for instance, the muscles of the three guinea-pigs, even though guinea-pig no. 3 was not killed until about a week after guinea-pig no. 1 (see Table I).

Few data have been published in which the vitamin B₁ content of animal tissues has been assayed in terms of International Units and directly against the International Standard. For roast chicken, raw beef and raw mutton the following values have been reported by Baker & Wright [1935]: 0.4, 0.5 and 0.6 I.U. per g. respectively. It is not known whether these tissues were "saturated" with vitamin B₁, but it is interesting to note that these values are not far removed from those observed in the "saturated" tissues described in this paper. The vitamin B₁ content of pig muscle appears to be unusually high when compared with that of other animal muscles so far examined, as it was reported by Baker & Wright [1935] to contain 3.2 I.U. per g.

SUMMARY

The maximum level of storage of vitamin B₁ in the muscle and the liver of the guinea-pig, fowl and pigeon has been studied. The highest concentrations in the muscles of the guinea-pig, fowl, pigeon and rat were found to be 0.3, 0.3, 1.2 and 0.6 I.U. per g. respectively. The corresponding values obtained for liver were 0.7, 0.7, 1.1 and 2.6 I.U. per g. respectively.

The author wishes to thank Dr L. J. Harris for his interest and valuable criticism.

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CLXXI. EFFECT OF SOIL TREATMENT ON THE VITAMIN B₁ CONTENT OF WHEAT AND BARLEY

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VARIOUS investigators have claimed that the nature of the manure applied to the soil influences the "vitamin B" content of wheat [McCarrison & Viswanath, 1926; Hunt, 1927] and grass seeds [Rowlands & Wilkinson, 1930]. Employing the bradycardia method of assay, Harris [1934] found that specimens of wheat from plots at the Rothamsted Experimental Station which had been treated with different types of manure did not show any striking variation in their vitamin B₁ content. Since then it has also been reported by other workers that differences in soil treatment did not affect the vitamin B₁ content of wheat [Scheunert & Schieblich, 1936] or of rye and barley [Scheunert & Wagner, 1937].

This note describes the results of further studies on the vitamin B₁ content of samples of wheat and barley harvested at Rothamsted in 1935 and 1936. The details of the soil treatment of the various plots are given in Table I.

Table I. *Details of soil treatment to the continuous wheat and barley plots at the Rothamsted Experimental Station*

Wheat Broadbalk Plot no.	Barley Hoosfield Plot no.	Type of manure
2	7-2	14 tons of dung per acre
3	6-1	No manure
5	4-0	Complete mineral manure: 3½ cwt. superphosphate 200 lb. sulphate of potash 100 lb. sulphate of soda 100 lb. sulphate of magnesia
7	4-A	Complete mineral manure plus 412 lb. of ammonium sulphate per acre
10	1-A	412 lb. of ammonium sulphate per acre

Method of assay. The bradycardia method [Drury & Harris, 1930; Birch & Harris, 1934] was employed. Comparative tests with graded doses of the International Standard (activated acid clay) were carried out simultaneously with these assays. A dose-response curve with the International Standard was constructed, from which the vitamin B₁ potency of the substance under test could be obtained by inspection [see Leong & Harris, 1937]. In assaying the flours a uniform dose of 2 g. of the finely ground meal was given to each test rat. The material was made into a thick paste with water before being administered.

Results. The results are detailed in Table II and depicted in Fig. 1. The tests on the whole wheat flours confirmed the previous observation of Harris [1934]

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in that there were no appreciable differences in the vitamin B₁ contents of the samples from the various plots. The values obtained for the 5 specimens of wheat flours ranged from 1.0 to 1.3 I.U. per g.

Table II. *Results of bradycardia tests on wheat and barley*

Material and plot no.	Period of cure of bradycardia, days (individual tests)	Mean	I.U. vitamin B ₁ per g.
Wheat, 1935:			
2	3.5, 3.5, 4, 4, 4, 4.5, 5, 6, 7, 8	5.0	1.2
3	3, 3, 4, 4, 4, 4.5, 5, 5, 5, 6	4.4	1.0
5	3.5, 3.5, 4, 4, 5, 5, 5.5, 7, 8, 8	5.4	1.3
7	3, 3.5, 4, 4, 4, 5, 5, 6, 6.5, 8	5.1	1.2
10	3.5, 3.5, 4, 4.5, 5, 5, 5.5, 6, 7, 8	5.2	1.2
Barley, 1935:			
7-2	5, 7, 8.5, 9, 9, 10	8.1	2.0
6-1	2.5, 3.5, 5, 5.5, 5.5, 6.5	4.8	1.1
4-0	4, 5, 5, 5, 5.5, 7	5.3	1.3
4-A	3.5, 4, 4.5, 5, 5, 6	4.7	1.1
1-A	2.5, 4.5, 5, 5, 5, 7	4.8	1.1
Barley, 1936:			
7-2	4, 4, 4, 5, 5, 5, 6	4.8	1.1
6-1	3, 3, 3, 4.5, 5, 5	3.9	0.8
4-0	2, 3, 3, 3.5, 5, 6	3.8	0.8
4-A	3, 3, 3, 4, 4, 5	3.7	0.8
1-A	4, 4, 4, 4.5, 5, 7	4.8	1.1
International standard, acid clay (mg.):			
10	2, 2, 2.5, 3, 3	2.5	—
20	3.5, 4.5, 4.5, 5, 5.5	4.6	—
30	4.5, 5.5, 6, 6, 8	6.0	—
40	6, 7, 7.5, 9, 10	7.9	—

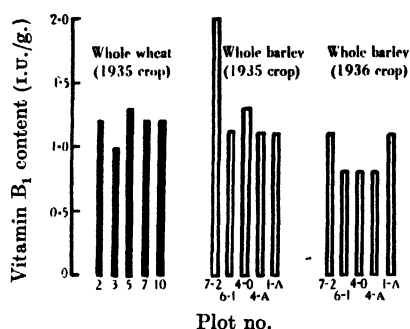


Fig. 1. Vitamin B₁ content of wheat and barley.

As regards the barley flours in the 1935 crop a higher vitamin B₁ content was found in the specimen from the plot treated with dung (2.0 I.U. per g.) than in the others, which gave an average value of about 1.2 I.U. per g. This test was again repeated with the barley crop obtained in 1936. On this occasion, however, no significant variation in the vitamin B₁ contents of the various plots was observed.

SUMMARY

The observation of Harris that the vitamin B₁ potency of wheat was not significantly influenced by soil treatment has been confirmed. Similar results were also obtained for barley.

My thanks are due to Sir John Russell and Dr Leslie Harris for their collaboration in this work.

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CLXXII. VITAMIN P

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SZENT-GYÖRGYI and his co-workers [Armentano *et al.* 1936] reported the presence in extracts of Hungarian red-pepper ("vitapric") and, later, in lemon juice of a substance other than ascorbic acid which could control the number of haemorrhages occurring in the course of certain clinical conditions. The clinical material upon which this original observation was made included three cases of vascular purpura, four cases of thrombocytopenic purpura, seven cases of infectious diseases, one case of myxoedema, and two cases of diabetes mellitus. In these cases the capillary fragility (resistance) was determined by the method of Borbély [1930] and the capillary permeability by an adaptation of the method of Landis, a detailed account of which is given [Armentano *et al.* 1936].

In two brief communications Bentsáth *et al.* [1936; 1937] claimed to have demonstrated by experiments with guinea-pigs the vitamin-like nature of this substance (or substances). As a result of this work they claimed to have determined the existence of a vitamin P which "brought back the fragile and permeable capillaries to their normal state" [Szent-Györgyi, 1937].

It was subsequently stated that the active material was flavanone in nature [St Rusznyák & Szent-Györgyi, 1936] and in a further communication by Bruckner & Szent-Györgyi [1936] it was claimed that the active fraction consisted of a mixture of hesperidin and eriodictyol glucoside. Contradictory statements as to the relative activities of these two fractions have been made [Bruckner & Szent-Györgyi, 1936; Szent-Györgyi, 1937].

The experiments on guinea-pigs were stated to show that the time of survival of animals on a scorbutic diet (Sherman-La Mer-Campbell) was prolonged from 28.5 days to 44 days by supplements of vitamin P given orally as 1 mg. daily of an active fraction prepared from lemon juice. It was further stated that at autopsy the animals which had previously received the vitamin P supplements showed fewer haemorrhages. Zilva [1937] and Moll [1937] have been unable to confirm the work on guinea-pigs, and even Szent-Györgyi [1937; 1938] has been unable to repeat the experiments.

Accordingly, since conclusions in support of the existence of vitamin P cannot be drawn from guinea-pig experiments, evidence for the existence of such a substance rests entirely upon the original clinical observations. The present author's considerable experience of the determination of capillary fragility in a large number of diseases in the human subject has shown that Szent-Györgyi's cases were unsatisfactory for the purpose. In vascular and thrombocytopenic purpura gross alterations in capillary fragility occur as a result of a series of events characteristic of these conditions and independent of the possible existence of vitamin P. The explanation of these variations will be given in another place; for present purposes it is sufficient to make the point that conclusions as to the efficacy of any substance in controlling bleeding or in decreasing the capillary fragility in purpura must be drawn with the greatest

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caution. Hence experiments on patients with purpura cannot be regarded as satisfactory in deciding the question whether there is a vitamin P.

During the last few years various workers have reported their inability to relate increased capillary fragility to states of vitamin C subnutrition. Attention has already been directed to this point by Scarborough & Stewart [1938] in whose paper appropriate references are given. In that preliminary communication the investigation of the capillary fragility in six cases of generalized vitamin deficiency was reported. It was shown that the oral administration of "hesperidin" could control the number of induced petechial haemorrhages in such subjects. In the present paper results of a more extensive investigation are presented. The purpose of the communication is to demonstrate the existence of a substance (or substances), present in fruits and extracts prepared from them, which does in fact control capillary fragility. A brief summary of the paper has already appeared [Scarborough, 1939].

EXPERIMENTAL

Clinical material. All subjects used in this investigation had abnormally increased capillary fragility. During the experimental period every case was the subject of a more or less severe degree of vitamin deficiency as a result of circumstances which are stated subsequently. It is held that the increased capillary fragility is an expression of this vitamin deficiency.

Test material. There is some confusion in the available literature regarding the nomenclature of the materials used in vitamin P studies and it is therefore desirable to describe briefly the nature of the material used in this investigation. Three different extracts containing the flavanones from certain fruit sources have been used:

Fraction C. A crude yellow powder, free from ascorbic acid, deposited spontaneously during the desiccation of Californian Valencia orange juice.

Fraction H. A colourless powder obtained from "C" by extraction with pyridine. Glaxo Laboratories Ltd. have generously provided these two fractions and I am indebted to them for the following account of the preparation of the material:

In a particular experiment 400 ml. of pyridine were used for the extraction of 36 g. of crude material. The dark brown solution was concentrated under diminished pressure to 100 ml. and this was then diluted with 1 l. of boiling water. After standing overnight 30 g. of slightly yellowish crystalline material were deposited. This crop was recrystallized twice more from a mixture of about 1 part pyridine to 5 parts water. The product so obtained consisted of colourless needles of m.p. 255–256°.

Both C and H are only slightly soluble in water. Accordingly, they were administered as a suspension in water in doses of 1.0 g. per day orally.

Fraction E.G. A solution (50 mg. in 1 ml.) of flavanones prepared from orange peel according to the method of Szent-Györgyi. It has been stated [Szent-Györgyi, 1938] that this material consists of a mixture of "eriodictyl glucoside" (a glycoside of 5:7:3':4':tetrahydroxyflavanone) and "hesperidin" (a glucoside of 4'-methoxyeriodictylol). Acknowledgement is due to Roche Products Ltd. for supplies of this preparation which is called by them "Citrin". Glaxo Laboratories Ltd. have also kindly supplied a similar material.

This fraction was administered intramuscularly in doses of 1–2 ml. per day (50–100 mg.).

Determination of capillary fragility. The capillary fragility (the ease with which

capillary walls burst in response to the application of pressure) was determined frequently, in the majority of cases daily, by one of two methods. A full account of the techniques involved in this determination is being prepared for publication. At the moment it is sufficient to explain that in the "positive pressure method" the fragility is determined in terms of the number of burst capillaries occurring during standard conditions in response to an increased intracapillary pressure, whereas in the "negative pressure method" the fragility is evaluated as the amount (in mm. Hg) of suction which is required to burst a single capillary loop, the suction being applied over a small area of skin. Several standard areas are used and these are appropriately numbered in the charts which follow. When the former method is used, improvement, that is to say, decreased fragility or an increased resistance, is indicated by a fall in the graph: in the suction method decreased fragility is indicated by a rise in the curve.

The charts given in this paper are typical examples; they are not to be regarded as representing isolated experiments.

Results

Exp. 1. Fig. 1. Negative pressure method

Female, aged 53. Nutritional anaemia; haemoglobin 9.3 g. per 100 ml. (58%); subsistence for over a year on a diet which on analysis was found to be deficient in first-class protein, minerals and especially vitamins. At point A the diet was adjusted to contain fresh fruit, including in particular three oranges daily. The effect on the capillary fragility in response to the consumption of fruit is shown.

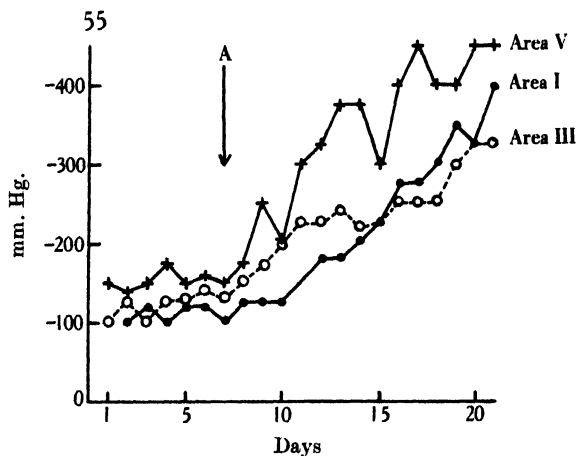


Fig. 1.

It is clearly possible that this improvement, which has been obtained in a number of individuals, may have been brought about by ascorbic acid. The next experiments bear upon this point. A second criticism is evidently that the coincident improvement in the anaemia may have occasioned the decrease in capillary fragility. In point of fact, however, in another investigation it has been found that an increase in blood haemoglobin concentration *per se* has no effect in decreasing capillary fragility.

Exp. 2. Fig. 2. Positive pressure method

Male, aged 56. Symptoms of generalized vitamin deficiency were occasioned by his having taken for many years a diet almost entirely lacking in fresh food. During the experimental period the subject was maintained on a similar diet and vitamin preparations were added one at a time. In spite of adequate doses of vitamins A (oral), B₁ (parenteral), C (oral) and D (oral) the capillary fragility

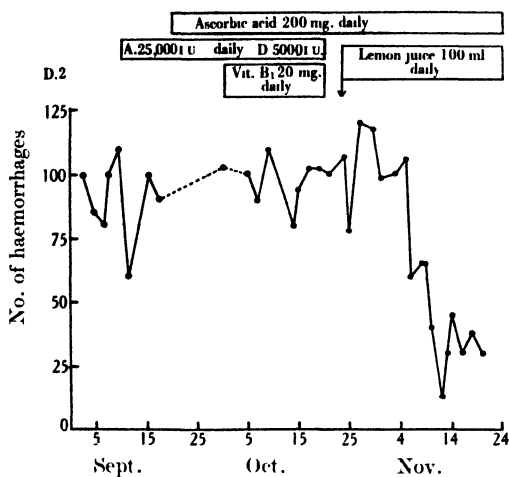


Fig. 2.

remained grossly increased. Lemon juice was now given as a supplement in doses of 100 ml. daily. The effect on capillary fragility is clearly shown. This experiment strongly suggests that lemon juice contains a substance (or substances) other than ascorbic acid which can control capillary fragility.

Exp. 3. Fig. 3. Negative pressure method

Male, aged 40. Dyspepsia; haemoglobin 13.6 g. per 100 ml. (85%). On account of his symptoms this patient had for 13 months been taking a diet of poor quality, deficient in fruit and vegetables. The capillary fragility was increased and remained high while he was taking the experimental diet (S. 1) which contains no fruit or vegetables. He was then given a diet (S. 2) differing from S. 1 in that it contained orange pulp and orange juice. It will be observed that while he was taking this diet the capillary fragility gradually decreased. Diet S. 1 was then substituted for diet S. 2 and ascorbic acid was given daily by mouth in amounts roughly equivalent to the vitamin C content of diet S. 2. In spite of the supplement of ascorbic acid the capillary fragility again increased. The flavanone fraction (E.G.) was then injected and a prompt and definite decrease in capillary fragility was obtained.

It was felt that this experiment was not completely satisfactory in that other unknown factors may possibly have contributed towards producing the initial increased capillary fragility. Furthermore, although the absorption of ascorbic acid was controlled by estimation of its elimination in the

urine, it is possible that ascorbic acid might have been effective had it been administered parenterally. Accordingly, an experiment was performed to test this point.

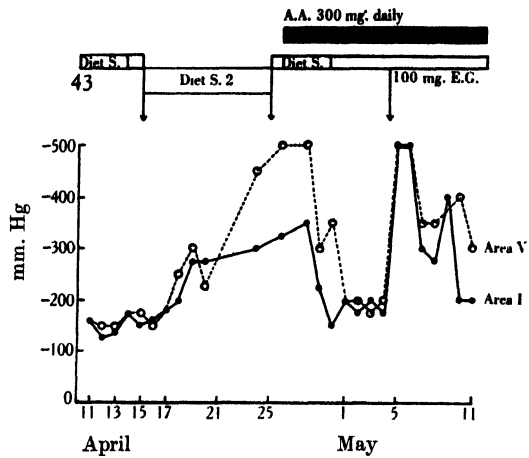


Fig. 3.

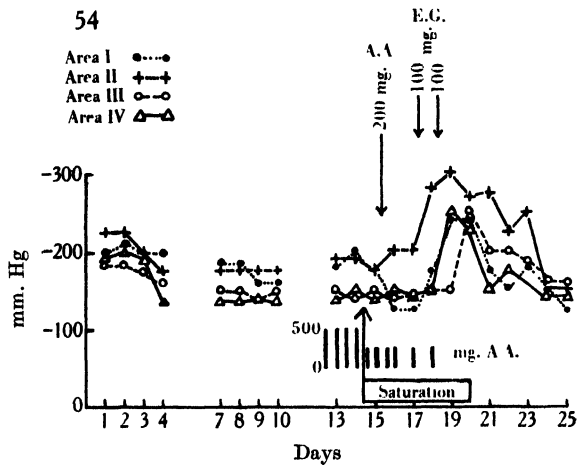


Fig. 4.

Exp. 4. Fig. 4. Negative pressure method

Male, aged 30. In this experiment an increased capillary fragility was induced in an otherwise healthy individual by placing him upon a diet containing neither vegetable nor fruit; all other food in it was twice cooked. Supplements of ascorbic acid were then added to the diet to the point of saturation (in the Harris and Ray sense) with no improvement in capillary fragility. 200 mg. of ascorbic acid were then injected intramuscularly without effect. 200 mg. of flavanone fraction (E.C.) were then given intramuscularly with the effect clearly indicated in Fig. 4.

Exp. 5. Fig. 5. Negative pressure method

Male, aged 50. Malignant stricture of the oesophagus resulting in marked interference with passage of food into the stomach. In such circumstances the increased capillary fragility is to be explained on the basis of a conditioned deficiency. As a result of treatment the subject became able to swallow liquids and accordingly 300 ml. of orange juice were given. There was a definite effect upon capillary fragility. Thereafter, 200 mg. of flavanone fraction (E.G.) were given in solution in 0.9% saline into the rectum with the result indicated in Fig. 5.

It will be clear from the above experiments that there is material present in orange and lemon juice which is capable of increasing the resistance of abnormally fragile capillary walls. This material is not ascorbic acid. It is present in flavanone fraction (E.G.) which is highly active when injected into human subjects suffering from pure or conditioned vitamin deficiency. It is now important to determine whether it be active by mouth.

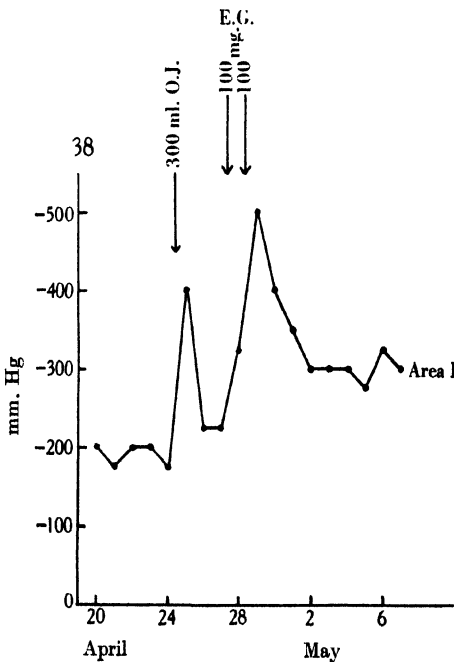


Fig. 5.

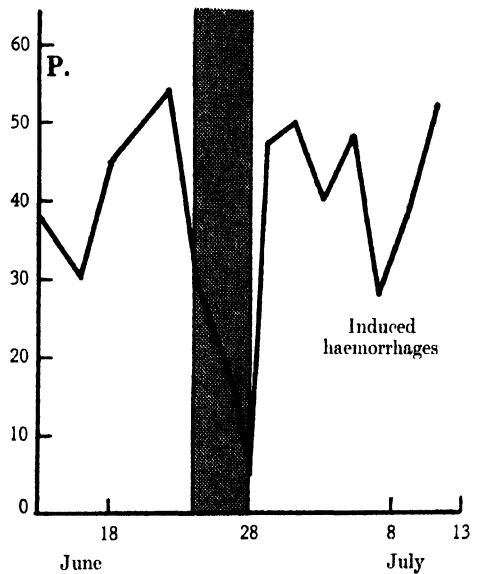


Fig. 6.

Exp. 6. Fig. 6. Positive pressure method

Female, aged 37. Vitamin deficiency on account of bad dietary habits. During the shaded period indicated in Fig. 6 flavanone fraction (C) was given by mouth in doses of 1 g. per day. Its effect in decreasing capillary fragility is evident.

Exp. 7. Fig. 7. Positive pressure method

Male, aged 58. Severe and generalized hypovitaminosis occasioned by economic and financial stringency. Flavanone fraction (H) was given orally in doses of 1 g. per day. The reduction in capillary fragility in response to the administration of flavanone material is again clear.

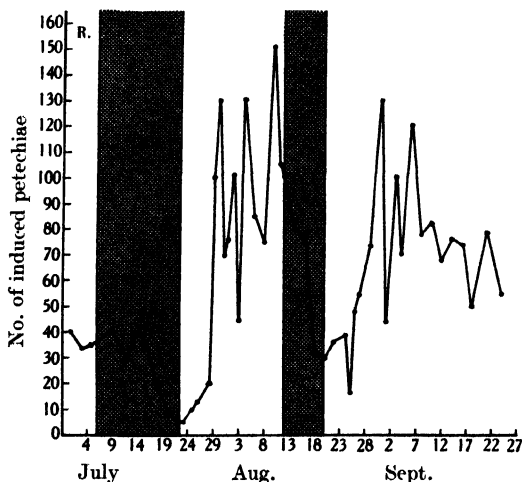


Fig. 7.

The latter two charts are to be regarded as typical of the results obtained in a series of 12 subjects.

Conclusions

In human subjects suffering from multiple vitamin deficiency of varying degrees of severity the capillary fragility was found to be increased. This increase has been determined in spontaneous, conditioned and induced deficiencies. There is present in orange and lemon juices, and in certain extracts made from them, a substance (or substances), flavanone in nature, which can increase the resistance of capillary walls to the application of pressure. The precise nature of the material producing this effect has not been determined. It is active when given by mouth, by intramuscular injection or through the rectum. The decrease in capillary fragility (or increased capillary resistance) has been produced in every case even when ascorbic acid, by mouth or by injection, has failed to produce this effect.

SUMMARY

The evidence upon which the existence of vitamin P is based has been reviewed and it has been shown that a conclusion as to the reality of such a vitamin cannot be maintained on the basis of the published work. Evidence is now adduced from experiments on human subjects which establishes the existence of a factor decreasing capillary fragility.

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CLXXIII. THE FERMENTATION PROCESS IN TEA MANUFACTURE

IV. TEA TANNIN AND ITS FERMENTATION PRODUCTS

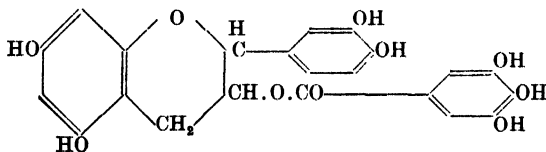
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INVESTIGATIONS into the nature and structure of tea tannin in both green leaf and made tea have occupied workers at Tea Experimental Stations in Ceylon, the Dutch East Indies, Assam, South India, Japan and Formosa for many years. Much of this work has remained unpublished until now, while the results which have appeared in the various journals are either mutually contradictory or must be taken to indicate the existence of a group of tannin bodies in the leaf, the relative proportions of which may vary considerably.

Working in Java, Nanninga [summarized by Deuss, 1914] obtained a crystalline product, $C_{20}H_{16}O_9$. Later Deuss [1923] reported the isolation of an amorphous product to which he ascribed the mol. wt. 404 and molecular formula $C_{20}H_{20}O_9$. Shaw [1930] and Shaw & Jones [1932] described a tea tannin preparation which was obtained by a method very similar to that previously elaborated at Tocklai and which will be described more fully below. Shaw's tannin seems very similar to that obtained by Deuss, and both preparations gave no gallic acid on hydrolysis with acids. Tsujimura [1930, 1, 2; 1931; 1935] obtained a crystalline tea tannin to which she assigned the structure



Later Deys & Dijkman [1937] obtained a similar crystalline product which liberated gallic acid on enzymic hydrolysis with a tannase preparation obtained from *Aspergillus niger*.

Other tea tannins have been reported, notably that of Oshima [1936] who considered an amorphous product he obtained to be bis-(5:7:3:4':5'-penta-hydroxy) flavpinacol. It is doubtful whether this claim can be substantiated as Russell & Todd [1934] have synthesized the corresponding 5:7:3':4'-tetra-hydroxy compound, which latter substance differs, principally in the colour of its aqueous solutions, from Oshima's tea tannin preparation.

Preparation and properties of tea tannin from green leaf

The standard procedure for the preparation of tea tannin at Tocklai is as follows.

500 g. freshly plucked leaf are boiled with $1\frac{1}{2}$ –2 l. distilled water for 1 hr. All damaged and diseased leaf is rejected; otherwise tannin oxidation and condensation products will be found in the final product. After cooling, the infusion is

filtered through a cotton wool plug. The filtrate is subjected to two extractions each with benzene and ether, and is then rapidly extracted two or three times with ethyl acetate. The united ethyl acetate extracts are dried over anhydrous Na_2SO_4 and then evaporated to 150–200 ml. on a water bath. The extract is then cooled and the tannin precipitated by adding 300–400 ml. CHCl_3 . The precipitate is filtered at the pump and dried *in vacuo*. The purity of this product as estimated by the Lowenthal and Hide powder methods usually varies from 80 to 90 %. The yield is only about 20–25 % of the total amount of tannin found in the leaf by analysis.

So prepared tea tannin cannot be classed as a true tannin. It is precipitated by nitrogenous bases such as quinine, and by Pb acetate, but it is not completely precipitated by gelatin except in the presence of acid and salt. Strong solutions are precipitated by FeCl_3 , and very dilute solutions give an intense colour reaction. This colour varies from green to an intense blue indicating that tea tannin may be either a catechol or a pyrogallol derivative or probably a mixture of both.

Prolonged acid hydrolysis never yields the slightest trace of glucose but in some cases gallic acid can be detected in the hydrolysate. As tea tannin is precipitated by Br water, gives the pine-shaving reaction for phloroglucinol and gives a rose pink precipitate (tannoform) on warming with formaldehyde and conc. HCl, it must be considered a condensed tannin and therefore derived from a catechin. The isolation in certain cases of gallic acid but not sugars from hydrolysates does not indicate the presence of gallotannins or hydrolysable tannins, and is explained satisfactorily by the presence of Tsujimura's product, the galloyl ester of epicatechin, in the tea tannin complex.

The nature of the tea tannin complex

From a consideration of the above properties of tea tannin preparations it follows that in all probability they are condensed tannins derived from both catechin and gallo catechin (3:5:7:3':4':5'-hexahydroxyflavan). The galloyl ester of epicatechin may or may not occur in this complex.

It is however a moot point whether the tannins of the green leaf are identical with the tannin preparation just described. According to Freudenberg [1933] the catechins undergo condensation on boiling in aqueous solution and it seems likely that this treatment, in the course of preparation, will modify the tannins originally present in the leaf. This belief is strengthened by the finding that the properties of the final product are to some extent affected by the method of extraction from the leaf.

The tea tannins obtained from cold acid extracts of leaf, using 5 % oxalic acid or 1 % HCl, show higher rotations than preparations obtained from the 1 hr. infusion with boiling water, as shown in Table I.

Table I

Extraction agent	$[\alpha]_D^{20}$
Boiling water	– 76.5°
1 % HCl	– 123.2°
5 % Oxalic acid	– 132.5°

Further, after extracting leaf with 5 % oxalic acid and salting out the extract, about 75 % of the tannin remained in the filtrate. On the other hand the greater part of the tannin is salted out from an aqueous infusion which has been boiled for 1 hr. These observations would accord with a greater degree of condensation of the tannin preparation from the boiled aqueous extract.

This latter preparation goes readily into aqueous solution, and is pure white in colour when freshly prepared. It has not the properties of a highly condensed substance. It would therefore appear likely that the tannins of the green leaf are not far removed from the parent catechins themselves. Small amounts of both *l*-epicatechin and galocatechin have been detected in the tea leaf, the former by Tsujimura [1929] and the latter by Oshima & Goma [1933]. These observations have been confirmed by several workers and a substance with all the properties of *l*-epicatechin was isolated by one of us [C. J. H., unpublished] as early as 1925.

It will therefore be adopted as a working hypothesis that the tannins in green leaf consist of a mixture of these two catechins, and probably of their simpler condensation products, together with the galloyl ester of *l*-epicatechin.

Analyses of amorphous preparations correspond closely with those expected for mixtures of the two catechins, whilst Nanninga's crystalline tea tannin approximates in its molecular formula to Tsujimura's product as the following table shows:

Table II

Tannin preparations	C	H	O
Nanninga crystalline	54.60	4.80	41.60
Deuss amorphous	59.97	4.96	35.07
Tsujimura crystalline	55.49	5.29	39.22
Epicatechin crystalline	62.07	4.83	33.10
Galocatechin crystalline	58.82	4.57	36.61

Condensations of tea tannin

According to Freudenberg [1933] catechins are converted into true tannins by condensation. This condensation is stated to take place between the secondary carbinol group in the pyran ring of one molecule and the phloroglucinol nucleus of another. As the condensation product still possesses the two necessary groups for further condensation the degree of complexity of possible condensation products is theoretically limitless. Such condensations of catechin according to Freudenberg take place on warming its aqueous solution, on treatment with acid or alkali, as a result of enzymic action or as a consequence of oxidation. The tannin bodies in green tea leaf undergo very similar changes to those of catechin.

If an aqueous solution of a tea tannin preparation is evaporated down to dryness a reddish-brown glassy product is obtained consisting of higher condensation products. Further, on heating a green tea leaf infusion under pressure at 100° the solution reddens and its Lowenthal tannin titre falls.

Condensations in acid solution. Acidification of a green leaf infusion to pH 1.2 produces no precipitate but decreases the Lowenthal titre of the solution. On boiling with acids however a tea tannin solution reddens and a red-brown precipitate is eventually formed. Deposition of this insoluble product increases with acidity and with the time of boiling, and after 24 hr. boiling with 5% H₂SO₄ no tannin is left in solution. At the same time gallic acid can be detected in the filtrate. Shaw [1930] found no gallic acid under the same conditions, which finding may indicate that Tsujimura's galloyl ester does not occur in the particular samples of South Indian leaf used by him. The tannin products precipitated in this way represent a whole range of substances whose solubilities in water, dilute acid and organic solvents vary considerably with the time of boiling. The presence of air does not seem to be necessary for the production of these products and consequently they are not considered to be the same as the tannin bodies in fermented tea in the formation of which oxygen is essential.

Effect of alkalis on tea tannin. In alkaline solution tea tannin behaves very similarly to other polyphenols with adjacent hydroxyl groups. In dilute alkaline

media a yellow coloration first develops deepening to light brown and finally to a dark brownish-black. In more strongly alkaline solutions the deepening of the colour is much more rapid.

The darkening in colour, with decrease in Lowenthal titre, will take place in slightly acid solutions; in fact tea tannin solutions are unstable at any pH greater than 5.0. At a higher pH the colour steadily darkens and the $KMnO_4$ titre using indigo carmine as an indicator decreases, as is shown in Table III where figures are given for the $KMnO_4$ titres of 10 ml. portions of tea tannin solutions at varying pH , kept at 25°.

Table III

pH	0 hr.	2 hr.	4 hr.	6 hr.	24 hr.
4.0	—	23.0	22.9	23.1	22.3
5.0	—	23.1	23.1	23.2	22.5
6.0	22.9	23.0	22.3	21.6	21.4
7.0	—	22.0	21.0	20.2	17.6
8.0 (phosphate)	—	22.0	21.7	—	17.0
8.0 (borate)	—	22.7	22.2	21.8	21.6

Titres are in ml. 0.04 N $KMnO_4$.

Browning of the solution and decrease in oxidizability are significant even at pH 6.0. A borate buffer was first used for the pH 8 value but here the change in 4 hr. was very slight. With a phosphate buffer at this pH browning was rapid. Borates were also found to inhibit the autoxidation of catechol and pyrogallol in slightly alkaline solutions. These phenomena are probably related to the enhancement of the acidity of boric acid by compounds, particularly sugars, containing the glycol group, and in that case are due to the formation of a boron-containing ring.

The tea tannin in alkaline solution at first retains its tanning powers and can precipitate gelatin from acid salt solution. Eventually however it loses its tanning properties. After 3 hr. in $N/10$ alkali at a temperature of about 25° the amount of oxidizable matter precipitated by gelatin in acid-salt solution is negligible.

The changes in tea tannin and other polyphenols in alkaline solution are associated with a considerable uptake of O_2 . At first both the rate and extent of O_2 uptake increase with increasing alkalinity, but in strongly alkaline solutions the rate only is affected by the strength of alkali. In $2N$ $NaOH$ O_2 uptake comes almost to a standstill within 10 min.

Assuming that the tannins in green leaf are a mixture of epicatechin, galocatechin and their simpler condensation products, the average mol. wt. of each tannin unit will be about 300. This figure was adopted in calculating the average O_2 uptake per mol. of tannin.

The O_2 taken up during alkaline autoxidation of various polyphenols was determined manometrically. 2 ml. portions of $M/100$ polyphenol solution were introduced into the main compartments of Warburg vessels and 1 ml. of approximately N $NaOH$ into the side bulbs. After attainment of temperature equilibrium the $NaOH$ was tipped into the polyphenol solution and uptakes recorded until they came to a standstill. The results expressed in atoms O per mol. of polyphenol are recorded in Table IV.

Each of the figures quoted is a mean of either three or four determinations.

The tea tannin contents of both the green leaf infusion and the tannin preparation were determined by Lowenthal's method. The discrepancy between the amounts of O_2 taken up under these varying conditions cannot at the moment be explained.

Table IV

Polyphenol	Atoms O taken up per mol.
Tea tannin (green leaf infusion)	6.6, 6.4, 7.1, 6.9, 6.4
Tea tannin (87 % pure preparation)	4.8, 4.7, 4.7
Catechol	4.7
Pyrogallol	3.1, 3.5
Gallic acid	4.7, 4.8
Phloroglucinol	0.2

The negligible degree of autoxidation of phloroglucinol and the close agreement between the total uptakes of other polyphenols and tea tannin preparations suggests that the alkaline autoxidation of tea tannin is a property of its catechol or pyrogallol nucleus alone.

Condensation during fermentation. In tea fermentation the tea tannin molecule is oxidized, and then as shown in the previous communication [Roberts, 1939, 2] undergoes further irreversible changes. Oxidized catechins are known to undergo condensation very readily. The properties of these oxidized and condensed products of tea tannin will be discussed after the changes undergone by tea tannin in the fermentation process have been described.

Fermentation changes in tea tannin

It was previously calculated [Roberts, 1939, 2] that each tannin molecule took up approximately 1 atom of O during the fermentation process. This calculation assumed the mol. wt. of tea tannin to be 442 and that about 60 % of the total O₂ uptake was accounted for by tannin oxidation. The figure of 1.08 ± 0.08 was the mean of six experiments only and with many more results available the average value became 1.4. If this is expressed in terms of an average mol. wt. of 300 per tannin unit the O₂ uptake comes very close to 1 atom of O per catechin molecule. Subtraction of the CO₂ outputs from the total O₂ uptakes to give the oxygen consumed in tannin oxidation is not completely satisfactory. Theoretically on the reaction scheme put forward for tea fermentation more and not less than 50 % of the total O₂ consumed should be used in carbohydrate oxidation. However we are dealing with a very much more complicated system than one of pure substrates and enzymes alone. The proteins, amino-acids and lipins are not likely to be entirely unaffected by the chain of reactions advanced to explain the fermentation process and, until these possibilities have been explored further, the above method of estimating tannin oxidation will be retained.

Oxidations of polyphenols by tea-enzymes. Two manometric methods are available for studying the oxidation of polyphenols by the enzyme system of the tea leaf. The minced tea leaf may be suspended in a solution of the polyphenol and the increase determined in total O₂ uptake over that shown by controls suspended in water alone. Alternatively the polyphenol may be added to the system when tannin oxidation has come almost to a standstill. In the latter case it is better to work with 100 mg. portions of minced tea leaf as with these smaller quantities of leaf, oxidation of tannin is relatively faster, and amongst other advantages enzymic inactivation by the products of reaction is likely to be less marked.

The total increase in O₂ uptake is not entirely due to polyphenol oxidation. The polyphenol may take the place of the tea tannin as an O₂ carrier and carbohydrate oxidation is thereby also increased. The ratio of CO₂ evolved to O₂ taken up is not very much altered by the addition of catechol or pyrogallol as is

shown in Fig. 1 which gives curves for O_2 uptake and CO_2 output for normal fermentation and in the presence of pyrogallol. (These curves do not represent the same sample of leaf.)

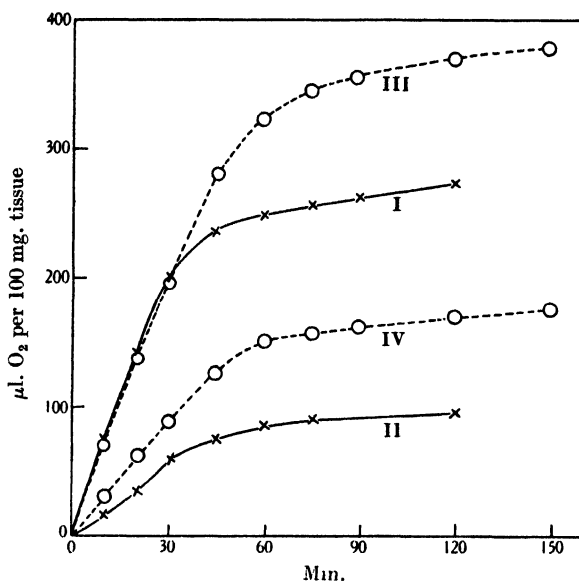


Fig. 1. Normal fermentation: I, O_2 uptake; II, CO_2 output; ditto + added pyrogallol; III, O_2 uptake; IV, CO_2 output.

Approximately, therefore, about 60 % of the increase in total O_2 uptake may be ascribed to the added polyphenol. Shaking must usually be prolonged to $2\frac{1}{2}$ hr. or more before the added polyphenols are nearly completely oxidized. Table V gives total uptakes with and without added polyphenol, and the calculated proportion of the increase due to polyphenol oxidation alone. In all cases with the exception of gallic acid the increase is almost equivalent to an uptake of 1 atom O per mol. polyphenol.

Table V

Substrate	mg. added	Total uptake control	Total uptake with polyphenol	Increase in uptake due to polyphenol oxidation	Theoretical uptake 1 atom O per mol. polyphenol
Catechol	1.10	543	689	88	112
		269	413	86	112
		152	288	82	112
Pyrogallol	1.26	269	455	111	112
		151	302	91	112
Gallic acid	1.70	383	497	68	112
Tea tannin (80 % pure)	3.50	156	304	89	104

From the similarity in uptakes between catechol and pyrogallol on the one hand, and tea tannin on the other, it may be concluded that the oxidation of the tea tannin molecule is restricted to the catechol or pyrogallol nucleus only.

The simpler polyphenols however are less rapidly oxidized by peroxidase than is tea tannin. It was shown in the previous communication in this series

[Roberts, 1939, 2] that addition of tea tannin to the system before oxidation commences prolongs the period where the rate of O_2 uptake is linear. The linear rate is not maintained any longer than for the normal period when either catechol or pyrogallol is added at the commencement of fermentation as is shown in Fig. 2.

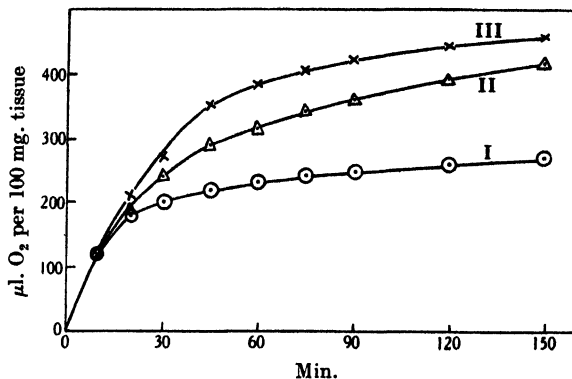


Fig. 2. I, normal fermentation; II, ditto + 1 ml. $M/100$ catechol; III, ditto + 1 ml. $M/100$ pyrogallol.

This effect is shown much better when the polyphenol is added when tannin oxidation is almost complete (Fig. 3). With tea tannin the initial rate of O_2 uptake is almost regained but the uptakes with pyrogallol and catechol are much slower.

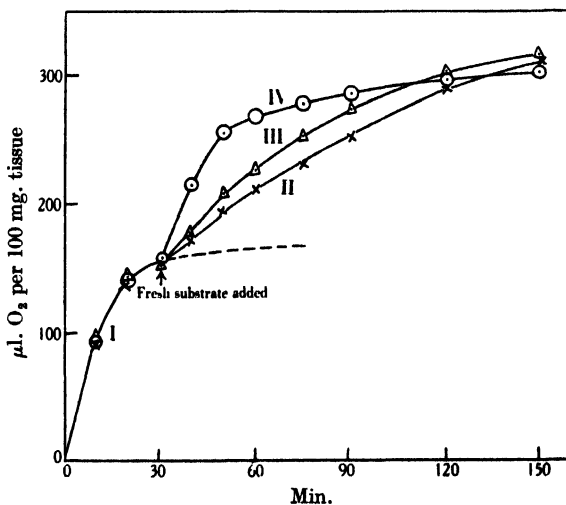


Fig. 3. I, normal fermentation; II, 1 ml. $M/100$ catechol added; III, 1 ml. $M/100$ pyrogallol added; IV, 1 ml. $M/100$ tea tannin added.

Gallic acid is oxidized even more slowly than catechol; in fact on occasions the increase in total O_2 uptake in the presence of gallic acid has not been significant. Using only 100 mg. of tea leaf tissue in each Warburg vessel the uptakes given in Table VI were recorded at 35° .

The influence of substitution in the catechol or pyrogallol ring on its efficiency as a peroxidase substrate finds a ready explanation in terms of the electron theory.

Table VI

	10	20	30	45	60	75	90	120 min.
Control	102	198	264	310	333	350	361	383 μ l. O ₂
1.70 mg. gallic acid added	98	181	244	312	362	401	433	497 μ l. O ₂

The O₂-ring in tea tannin is strongly cationoid and acts therefore as a proton repeller. Consequently the loss of 2H atoms to form an *o*-quinone is much easier in a catechin or other flavan derivative than with the simpler polyphenols. Conversely in gallic acid the carboxyl group is strongly anionoid and *o*-quinone formation is correspondingly more difficult.

As flavans such as catechin and, probably, quercetin are more rapidly oxidized by peroxidase than the simpler polyphenols such as catechol, it is not surprising that the former are the more generally found in plant tissues where they function as O₂ carriers in the respiratory process.

The condensation changes in tea tannin in fermentation

Two methods have been used in the past to study the changes taking place during the fermentation of tea. In the manometric method O₂ uptakes and CO₂ outputs were determined, while in the titrimetric method, as described in an earlier paper [Roberts, 1939, 1], the falls in the KMnO₄ titres of both water-soluble tannins and non-tan oxidizable matter were followed. By a comparison of these two methods it is immediately obvious that the fall in the total KMnO₄ titre (tannins + non-tans) is greater than would be expected if this fall were due to oxidation alone. In Table VII are recorded total O₂ uptakes at about 25° for fermenting leaf in μ l. per mg. dry wt. of tissue compared with the decrease in KMnO₄ titre for tannins and non-tans also expressed in terms of μ l. of O₂ per mg. dry wt. The uptake of O₂ by leaf crushed in the rollers is slower than in the Warburg vessel. When the bruised leaf is spread about 1 in. thick on the fermenting surface the rate of diffusion of O₂ into the tissues is the limiting factor which decides the rate at which fermentation proceeds. The rate at which O₂ is taken up in this process may be measured by manometric determinations, at intervals, of the total amount of O₂ taken up by finely-minced samples of partly fermented leaf, and subtracting these from the total uptake of fresh leaf after mincing. O₂ is taken up by leaf fermenting under factory conditions at rather less than half the rate of similar leaf finely minced and suspended in Warburg vessels. This is conclusively shown by comparing the manometric figures below with those plotted in Fig. 6 where the uptake exceeds 8 μ l. per mg. dry wt. after 30 min. shaking at 25°.

Table VII

	Fermented				
	Rolled	1 hr.	2 hr.	3 hr.	25 hr.
Manometric	1.53	6.24	9.49	9.95	11.55
Lowenthal: total KMnO ₄	3.4	8.6	14.5	17.9	24.7
non-tan	0.6	2.4	3.6	5.7	6.0
tannin	2.8	6.2	10.9	12.2	18.7

The figures represent μ l. O₂ per mg. dry wt.

Throughout the whole fermentation process (usually interrupted after 2-3 hr.) the decrease in oxidizability as measured by KMnO₄ titres is greater than would be expected from the amount of O₂ taken up. The decrease in non-tan oxidizable matter is just about equivalent to the normal CO₂ output so that

it is the tannin whose oxidizability decreases so much more than would be expected from the amount of O_2 consumed in its oxidation.

This decrease in tannin titre cannot be entirely accounted for by a less complete extraction of the tannins from the fermented leaf. As will be shown later part of the tannins of fermented leaf seem to combine with leaf-proteins and are no longer soluble in hot water, but the proportion of water-insoluble tannin is not large enough to account for the discrepancy under consideration. It was shown by Roberts & Sarma [1938] that in the oxidation of tea tannin in aqueous solution by peroxidase and H_2O_2 , the decrease in tannin titre was greater than the equivalent amount of H_2O_2 used up in the oxidation. In this case the whole of the tannin after oxidation remains in solution.

In Part II of this series [Roberts, 1939, I] it was shown that no O_2 uptake was recorded in the oxidation of tea tannin by H_2O_2 and a peroxidase preparation, which agrees with the findings in this communication that 1 atom O only is consumed by each mol. of tea tannin. Some change, which lowers the $KMnO_4$ titre of tea tannin, must therefore occur in addition to ordinary oxidation, and it seems almost certain that this change must be a condensation.

It has always been observed that the curve obtained for the decrease in the tannin titre during fermentation is not continuous (see Fig. 2 in Part II and Fig. 4 in Part III of this series) but shows a point of inflexion. Such a curve usually indicates that the changes measured are the summation of two or more consecutive changes. The general shape of the O_2 uptake curve is known and one would expect the rate of condensation to follow a sigmoid curve. The summation of both can give a curve similar in character to that normally observed.

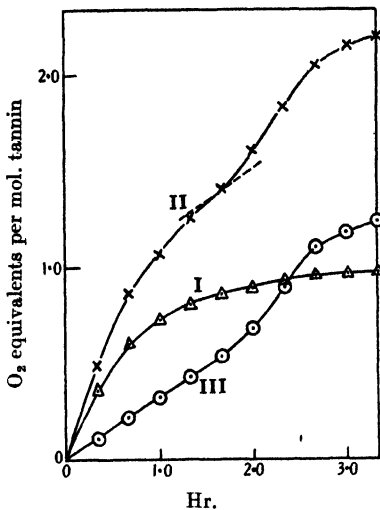


Fig. 4. I, normal O_2 uptake curve; II, decrease in tannin titre during fermentation; III, rate of condensation by difference.

Influence of temperature on fermentation

At higher temperatures of fermentation there is a greater decrease in both tannin and non-tan $KMnO_4$ titres as shown in Fig. 5 where curves for the decreases in these values at 38° (100° F.) and 21° (70° F.) are shown.

An increase in temperature results in accelerated O_2 uptake and a greater total uptake as shown in Fig. 6. The greater total uptake however is due to increased carbohydrate oxidation as the carbohydrate in the leaf, unlike the tannin, is not all oxidized in the fermentation process. Although the initial rate of tannin oxidation (O_2 uptake- CO_2 output) is accelerated by increasing the temperature, the total amount of tannin oxidized remains the same, as shown in Fig. 7.

Although at higher temperatures oxidation cannot proceed beyond an uptake of 1 atom O per molecule of tannin, the condensation process is not limited in any such way. Presumably the higher the temperature of fermentation the more highly condensed are the products of reaction, and the greater the decrease in the $KMnO_4$ titre.

From 25 to 40° the rate of the enzymic reactions in the fermentation process increases by only about 20 %. This very low temperature coefficient is an indication that the enzymes are working at very nearly their maximum temperature and that inactivation would follow any further increase in the temperature. On the other hand the temperature coefficient of condensation is likely to be normal, so that an increase in temperature from 25 to 40° while increasing the rate of

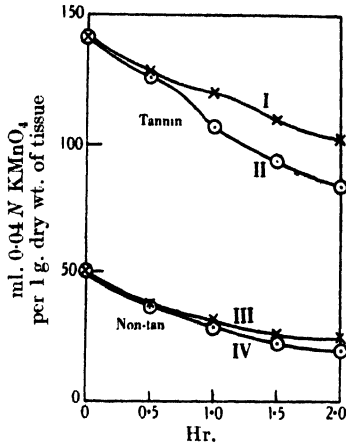


Fig. 5. Tannin and non-tan titres during fermentation at different temperatures. I and III, 21° II and IV, 38°.

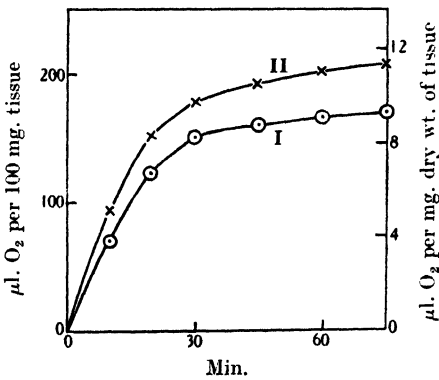


Fig. 6.

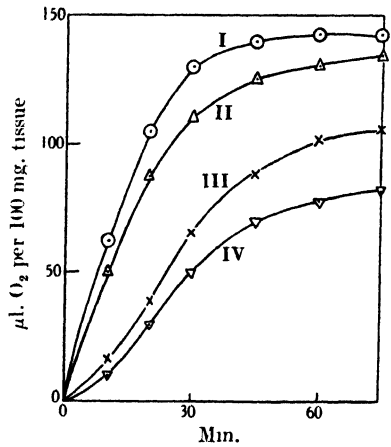


Fig. 7.

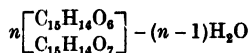
Fig. 6. Normal O_2 uptake in fermentation: I, 25°; II, 40°.

Fig. 7. Variation of tannin and carbohydrate oxidation with temperature. I, tannin oxidation at 40°; II, tannin oxidation at 25°; III, carbohydrate oxidation at 40°; IV, carbohydrate oxidation at 25°.

tannin oxidation by only 20 % increases the rate of condensation by about 150 %. A marked difference in the characters of infusions of teas fermented at these two temperatures would therefore be expected. In confirmation of this Benton [1939] has shown that there is an inverse correlation between quality, and temperature of fermentation, at temperatures of 70° F. and over.

The tannins of made tea

The tannins of green tea leaf are themselves a fairly complex mixture but the tannin bodies of made tea are very much more so. Under normal manufacturing conditions oxidation of the tannins is about 80–90 % complete and the oxidized tannins are present as a huge range of condensation products. It seems unlikely at present that these products will be such well-defined substances as the proteins or carbohydrates, and in the general formula



for condensed tea tannins, n may apparently have any value.

The isolation of definite compounds, to which various characters of the tea infusion may be attributed, does not therefore seem likely to be very successful. All that can be hoped for is a separation of the tannin bodies into fractions of greater or less pungency, colour and other such qualities. This has to a certain extent already been achieved. Tannin bodies in made tea are precipitated by acid and by salt, and apparently the ease of precipitation increases with the extent of condensation. Solutions of white tea tannin or green leaf infusions are not precipitated by dilute acids whereas an infusion of made tea gives a precipitate when acidified to 1 % by H_2SO_4 . After acidification, saturation with salt precipitates a further quantity of tannin bodies. The filtrate after the two precipitations is a clear golden colour and some unchanged white tannin can be obtained from it by extraction with ethyl acetate.

The precipitates obtained are still oxidizable by the Lowenthal method but not to the same extent as is the tannin in a green leaf infusion.

Precipitated from	g. tannin oxidized by 1 ml. N KMnO_4
White tannin	0.0416
Acid precipitate	0.1365
Acid-salt precipitate	0.1092

The reducing powers of the precipitated red tannin substances are much lower than that of white tannin, which is to be expected as they are formed from the tannin of the green leaf by processes of oxidation and condensation.

Judging by the high Lowenthal factor of the acid-precipitated tannin, the increase in the acid-precipitable matter as fermentation proceeds [Evans, 1930] and the light colour of the filtrate it is likely that this fraction represents some of the more highly condensed tannins which are responsible for the dark colour of the infusion. Too great a proportion of this fraction might be expected to produce a liquor remarkable for its depth of colour alone and lacking in the astringent character due to the presence of less highly condensed products. This expectation is borne out by the correlation found by Evans [1929] in Ceylon between valuation and acid-soluble tannin on teas from any one estate. If quality of leaf and conditions of manufacture are reasonably constant the acid-soluble tannin content seems largely to determine the value of the made tea.

According to Freudenberg [1933] the condensation products of catechin have quite marked tanning powers. In the case of tea it was established by Carpenter & Harler [1932] that whereas the unoxidized tea tannin of a green leaf infusion gave only a cloudiness with gelatin, a considerable proportion of the tannin in a made tea infusion is precipitated by gelatin alone.

One would expect from the above considerations that tea tannin would have a greater tendency to combine with the leaf proteins after fermentation. Green

leaf after repeated extractions with hot water leaves a residue which on treatment with alkali gives a light brown extract while at the same time there is a small O_2 uptake. With made tea three successive extractions for 1 hr. with boiling water remove all the water-soluble tannin but the spent leaf still has a brownish colour. The brown pigment may be dissolved out by alkali. There is a considerable darkening in colour of the alkali extract and manometrically a large O_2 uptake may be demonstrated. If the uptake of O_2 be assumed to give an approximate measurement of the tannins present a comparison of this uptake with those of the various extracts will give an idea of the proportion of the tannin insoluble in water. The results of one such experiment are recorded below.

Table VIII

	Total O_2 uptake per mg. dry wt. of original tea μ l.
First aqueous extract	34.0
Second and third aqueous extract combined	4.9
Residue	10.0

Some 20% of the total tannins are apparently insoluble in hot water and it seems quite likely that this amount is in chemical combination with the proteins of the leaf. Alternatively the water-insoluble tannin in this case may be too highly condensed to be readily soluble in water.

As fermentation proceeds there is a decrease not only in the Lowenthal titre but also in the proportion of water-soluble solids in the made tea. A highly significant correlation exists between these two values. The greater part of the decrease in water-soluble matter can be attributed to a diminished solubility in water of the tannin bodies.

SUMMARY

The preparation and properties of tea tannin are described. Tea tannin in green leaf probably consists of a mixture of *l*-epicatechin, gallo catechin and their simpler condensation products. The galloyl ester of epicatechin is present in some types of leaf only. Tannins in made tea are much more complex.

In the fermentation process the catechol or pyrogallol nucleus only becomes oxidized. The oxidized tannin then undergoes extensive condensations. At higher temperatures of fermentation (40°) the temperature coefficients of the enzymic processes are lower than that of the condensation process; consequently teas manufactured under such conditions contain more highly condensed tannins.

Strongly cationoid polyphenols such as tea tannin are readily oxidized by peroxidase while the anionoid gallic acid is oxidized slowly.

The authors are grateful to Mr P. H. Carpenter, Chief Scientific Officer, for his continued interest in the work, and to the Indian Tea Association for permission to publish these results.

Acknowledgement must also be made of the high standard of technical assistance throughout the course of this work rendered by Mr P. B. Sen Gupta and Mr S. N. Sarma.

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CLXXIV. THE QUANTITATIVE SEPARATION OF THE SKELETON OF SMALL ANIMALS

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(Received 13 July 1939)

STUDIES of the effects of dietary changes on the inorganic composition of the skeleton and on the rate of deposition of substances in the skeleton have been impeded by the difficulties involved in its complete separation from the soft tissues. Although dissection can always be employed as a final resort, the procedure is tedious and time-consuming and the danger of loss of small bones or bone fragments is always present. While it is sufficient simply to analyse the leg bones in certain types of experiments, other investigations require information of a different kind and to such cases the present technique has been found well suited. Comparisons are generally between litter mates of the same sex. The method can also be advantageously used in cases where pathological calcification of the soft tissues occurs, in which case total body analyses are very misleading.

Methods are on record which depend upon hydrolysing the flesh with boiling alkali or glycerol-alkali. It was felt that such methods are open to criticism since there is the danger that calcium, magnesium and phosphorus from the soft tissues may precipitate under such conditions and lead to erroneous results. This is of even greater importance in experiments where pathological calcification of the soft tissues is found. It seemed advisable to resort to less drastic treatment.

The present communication describes the conditions under which the skeleton can be obtained free from other tissue by means of proteolytic enzymes. The method has been applied only to the rat and all details given refer to that animal.

Analytical methods. The methods of analysis applied to aliquots of the dilute HCl ash extracts were:

Calcium	Clark & Collip [1925]
Magnesium	...	Godden & Duckworth [1935]
Phosphorus	...	Fiske & Subbarow [1925]

METHOD

The method consists of incubating the partially cleaned skeleton with either papain or trypsin. Both enzymes have given satisfactory results, papain being somewhat more rapid and thorough in action than trypsin. However, we have been unable to obtain papain samples which were not highly contaminated with Ca and, although this did not appear to affect the values we obtained, we have felt that the use of the much purer trypsin was probably preferable.

The value of buffers in maintaining a fairly constant pH was investigated, but this added refinement was abandoned. The use of phosphate buffers was obviously excluded and such others as were tested showed a tendency (more or

less marked according to the buffer used) to remove inorganic matter from the skeleton. Moreover, very little, if any, advantage was gained by using buffers.

In cases where papain was employed, marked acceleration of the action was observed after activation of the preparation by a few drops of 3 % HCN.

The technique finally adopted is as follows: after killing the animal by gas, the hide, viscera and intestinal tract are removed and the carcass placed in boiling distilled water for 2 min. Such portions of the flesh as can then be easily dissected are removed and the skeleton is then placed in a beaker and 100–200 ml. of 0.5 % trypsin are added. The beaker and contents are then placed in an incubator and digested for 4–6 hr. at 37° with hourly stirring.

In cases where papain is used, better results are obtained if incubation is carried out at 50°. At the end of the period of incubation the liquid is poured off the skeleton and the bones are washed several times with hot distilled water in order to remove small fragments of tissue. Care must be exercised to ensure that none of the smaller bones are lost during decanting. Disarticulation is fairly complete except in the case of the spine; the spinal cord is still intact and must be dissected out. The brain also requires separate removal.

RESULTS

For the purpose of testing the method, it was decided to use the left and right femora of rats. A series of such comparisons was first made in which both of the bones were cleaned by mechanical dissection in the usual way. The results are given in Table I and, except in the case of one animal (rat no. 4), show a very close agreement between the two sides. The values for rat no. 4 show a marked variation for the weight of ash as well as for the weights of Ca and P in the ash. An occasional animal showing such differences has been found in similar tests in other experiments.

Table I. *Comparison of the composition of the left and right femora after mechanical cleaning*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
1	Left	86.6	44.7	17.1	8.4
	Right	87.7	45.1	17.3	8.4
2	Left	136.6	78.4	30.4	14.3
	Right	138.0	77.8	30.4	14.2
3	Left	136.1	79.8	31.0	14.3
	Right	138.2	79.7	31.2	14.5
4*	Left	239.7	140.4	54.8	25.3
	Right	271.1	158.2	60.6	28.0
5	Left	246.5	146.6	55.6	26.3
	Right	250.2	145.5	55.2	25.7
6	Left	282.1	171.8	65.2	30.7
	Right	278.8	170.6	65.6	30.1

* It will be noted that there was a large difference between the two femora of this rat. This difference was quite obvious at the time of dissection.

Table II gives the results for a similar comparison in which the left femur was cleaned by mechanical dissection and the right femur by digestion with papain. Table III shows similar data for a series in which the right femur was cleaned by digestion with trypsin. The agreement between the data for the two sides for

Table II. *Comparison of the methods of cleaning the femora; left femur, mechanically cleaned; right femur, digested by papain*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
7	Left	93.6	46.2	17.0	8.7
	Right	86.5	44.8	16.8	8.5
8	Left	87.2	43.3	16.0	8.0
	Right	74.3	41.7	15.7	8.0
9	Left	149.6	77.2	28.6	14.2
	Right	129.8	74.9	28.2	14.2
10	Left	149.2	78.3	29.2	14.4
	Right	131.2	75.6	28.2	14.1
11	Left	191.0	118.4	45.0	21.2
	Right	189.3	118.3	45.4	21.2
12	Left	214.3	128.3	48.4	22.8
	Right	203.0	128.4	49.6	22.4

Table III. *Comparison of the methods of cleaning the femora; left femur, mechanically cleaned; right femur, digested by trypsin*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
13	Left	105.3	57.3	21.4	10.6
	Right	94.4	54.9	20.8	10.0
14	Left	100.0	56.2	21.0	10.1
	Right	93.7	56.0	21.1	10.1
15	Left	149.0	86.0	32.6	15.6
	Right	139.5	85.1	32.6	15.6
16	Left	152.3	88.2	33.8	15.8
	Right	145.6	88.7	34.2	15.8
17	Left	303.5	183.7	70.0	33.3
	Right	288.1	182.6	70.0	33.1
18	Left	313.2	187.9	71.2	33.6
	Right	304.1	187.0	71.6	33.6

Table IV. *Ca and P losses from bone during digestion*

Wt. of bone mg.	Ca mg.	P mg.
1571	0.57	0.20
1607	0.56	0.20

individual rats is remarkably close. The root mean square of the differences between the left and right ash values in the case of mechanical cleaning is 0.8, in the case of papain treatment 1.7 and in the case of trypsin treatment 1.2.

As a further check, the fore and hind leg bones of two rats were mechanically cleaned, air-dried and weighed and then incubated for 6 hr. with 0.5% trypsin solution. The amounts of Ca and P passing into solution were then determined and the amounts, as shown in Table IV, were found to be practically negligible.

The combined data show that in all cases the differences and the slight losses obtained are not significant in terms of the total ash or total amount of each element in the skeleton and fall within the range of analytical error.

It is clear that larger differences arise in the weights of the dry fat-free femora as a result of the enzyme treatment. This is undoubtedly due to the

proteolytic action of the enzyme on the organic matrix of the bone. Data obtained by this method for organic residue would, therefore, be false. This does not, however, apply to the inorganic constituents investigated, which are not appreciably affected.

Since the above studies were completed, it became necessary, for the purpose of another investigation, to determine the Mg content of the entire skeleton of rats. As a check, the skeleton and the right femur were cleaned by the use of trypsin and the left femur by mechanical dissection. The root mean square of the differences between the weights of Mg found in the two legs was 0.06 mg., the average total value being 2.06 mg.

SUMMARY

A proteolytic method of separating the entire skeleton of the rat for the estimation of Ca, Mg and P is described.

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CLXXV. URINARY OUTPUT OF CREATINE AND CREATININE ASSOCIATED WITH PHYSICAL EXERCISE, AND ITS RELATIONSHIP TO CARBOHYDRATE METABOLISM

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(Received 24 July 1939)

THE first accurate quantitative estimations of creatine and creatinine were carried out by Folin [1904]. Since that time it has become generally accepted that creatinuria occurs regularly in both sexes during childhood and also during menstruation and pregnancy. It is also known to occur in such conditions as complete starvation, diabetes, Graves' disease and certain myopathies. Apart from such conditions as these, creatinuria has not been generally accepted as occurring in adult men. Thus Rose [1911-12] and Harding & Gaebler [1922] regard 16 years of age as the limit for creatinuria, whilst Light & Warren [1934] found creatinuria in males only up to the age of 19. Recently, however, it has been reported that creatinuria may occur under certain conditions at later ages. Djen & Platt [1934] found creatinuria in adult males of Chinese extraction, whilst Taylor & Chew [1936] described creatinuria, increasing after exercise and diminishing on resting, in students and laboratory workers between 20 and 34 years of age. Haldi & Bachman [1936] found that the ingestion of sugars, glucose and particularly fructose, produced a creatinuria which was further increased by exercise.

Folin [1905] studied the creatinine output in a number of "normal" individuals and noted that the daily creatinine excretion for a given individual was constant and independent of the amount of nitrogen excreted in the urine (the amount varying from 1050 to 1660 mg. per day). At this time he proposed the term "creatinine coefficient" to indicate mg. creatinine excreted daily per kg. body weight. It appeared that the more obese the individual the lower the creatinine coefficient. Shaffer [1908, 1] noted that the daily creatinine output in a large number of individuals seemed to be directly proportional to their muscular development. He also defined the creatinine coefficient in terms of mg. creatinine, or creatinine nitrogen, excreted daily per kg. body weight.

To obtain further information on these points, particularly the influence of age, muscular activity and carbohydrate intake, the daily excretion of creatine and creatinine in a group of students attending the Carnegie Physical Training College, Leeds, was investigated. These men, ranging in age from 19 to 30, were carrying out a fixed routine of physical activity including gymnastics, swimming, athletics and team games, extending over 9 months. The diet was such that the average daily supply of food amounted to 4200 calories per head. It contained large amounts of readily assimilable carbohydrate and appeared to be adequate from the point of view of its content of animal protein and vitamins. Because of the large amount of carbohydrate being metabolized by the subjects, and in view of the important role played by vitamin B₁ in intermediate carbohydrate metabolism, special attention was paid to the adequacy of the supply of this

vitamin. The diet was apparently adequate for the supply of energy, since the majority of students showed an increase in weight during the year.

Observations were made on 97 students who were dealt with in two groups. Group A consisted of 42 students on whom observations were made during October, November and December 1936. Group B consisted of 55 students on whom observations were undertaken during October, November and December 1937, and repeated in May and June 1938. In addition, a sub-group of 12 individuals was taken from Group B and fed for a period on a low carbohydrate diet, and another sub-group of 12 individuals was taken in December 1939 to determine the effect of exercise on creatine and creatinine output.

EXPERIMENTAL

Great care was taken in the collection of urine to ensure that an accurate 24 hr. sample was obtained. Urine was voided at 8.0 a.m., this sample being thrown away; thereafter all samples voided were collected, including a final one at 8.0 a.m. the following morning. Urine was collected at all times immediately before going to stool. A few drops of toluene were added to each collecting bottle.

Estimation of creatine and creatinine. Preliminary tests for acetone bodies and reducing sugar were carried out by Rothera's and Benedict's tests, since both acetone and glucose interfere with the accurate estimation of creatine. This is a precaution which appears to have been overlooked by a number of workers.

Quantitative estimation of creatinine was carried out by the micro-method of Folin and creatine was estimated by the modification of Folin's micro-method, described by Hunter [1928].

RESULTS

Creatine excretion

The examination of a 24 hr. sample of urine from 97 subjects demonstrated creatinuria in 96 of them. The daily output of the 96 varied from 92 to 1200 mg., the mean being 637 mg. The results are expressed graphically in Fig. 1.

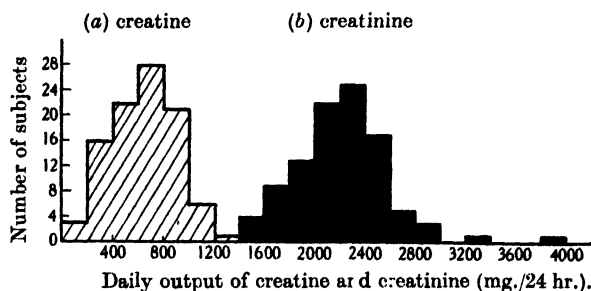


Fig. 1. The daily output (mg./24 hr.) of creatine (a) and creatinine (b) as observed in 97 subjects.

The present investigation showed a higher daily excretion of creatine in males than has hitherto been recorded, and it was surprising to find creatinuria in all cases examined except one, since most previous observations on normal adults indicated a complete absence of creatine from the urine. It appeared likely from the findings of other workers that one or both of two factors might be responsible for this high output: (a) high carbohydrate intake, which would accord with the findings of Haldi & Bachman [1936]; (b) hard intermittent muscular work, sufficiently severe to lead to oxygen debt several times a day.

Accordingly, it was decided first to investigate the effect of a low carbohydrate diet on the daily excretion of creatine.

A group of 12 individuals from Group B was placed for 1 week on a diet as low as possible in readily assimilable carbohydrate. All jam, sugar, honey, marmalade, cakes etc., were entirely eliminated and bread and potatoes cut down to a minimum. The diet consisted largely of meat and vegetables and the intake of these was increased to try and maintain the total calorie intake at the usual level. Unfortunately it was found impossible to estimate the calorie intake but none of the subjects showed a positive test for acetone bodies while they were on the diet. While living on this diet it became more difficult for the subject to perform his routine physical activities, fatigue came on early during the performance of work and there was a general disinclination to perform the various physical activities demanded of him. One individual collapsed and had to rest in bed. As can be expected, there was some difficulty in persuading the subjects to adhere strictly to the diet, particularly as they appeared to have an actual craving for sweet things. It is remarkable that normally a large amount of their diet was supplied by readily assimilable forms of carbohydrate, taken in the form of jam, sugar and honey. The daily excretion of creatinine and creatine was determined immediately before commencing the new diet, during the first day and during the fourth day. Observations at the end of the week were nullified by the inability of several individuals to adhere to the diet.

Table I. *Creatine and creatinine outputs of 11 individuals on a low carbohydrate diet*

High carbohydrate diet		Low carbohydrate diet 1st day		Low carbohydrate diet 4th day	
Creatinine mg.	Creatine mg.	Creatinine mg.	Creatine mg.	Creatinine mg.	Creatine mg.
2330	640	2446	290	1800	0
2000	750	2220	348	2053	370
1860	720	1974	628	1740	300
1780	615	2120	395	2116	380
2370	890	2040	464	2687	395
2040	270	1980	155	2052	230
2520	160	2160	464	2208	460
2096	880	2180	476	2134	0
2450	740	2225	314	2040	0
2220	1440	2000	880	2026	120
1870	590	2200	430	2320	300
Mean 2139	700	2140	440	2107	230

The results of the experiment are given in Table I and show a definite lowering in the daily excretion of creatine in all cases but one. In three cases the creatinuria had disappeared by the fourth day. This was in marked contrast to the findings in the 97 individuals who were assimilating a high carbohydrate diet, among which only one showed an absence of creatine. From calculations of the mean values and the standard deviations it can be shown that the results are statistically significant.

The explanation of these results was complicated by the fact that in addition to the decreased intake of carbohydrate there was an unavoidable decrease in the amount of physical exercise performed. In order to assess the effect which this decrease in activity might have had on the results, a control experiment was carried out to find the effect of decreased exercise on the excretion of creatine.

The excretion of creatine was determined in a group of 12 subjects on Thursday, Saturday and Sunday of one 7-day period. Although there was a considerable decrease in activity on Saturday and Sunday compared with Thursday, the small decrease noticed in the mean excretion of creatine on Saturday and Sunday was without significance and some subjects actually showed an increased creatinuria (see Table II).

Table II. *Effect of reduced amount of physical exercise (Saturday and Sunday) on the creatine and creatinine output of 12 subjects*

Thursday		Saturday		Sunday	
Creatinine	Creatine	Creatinine	Creatine	Creatinine	Creatine
2194	760	1960	476	1910	554
2320	1749	2230	321	2304	444
1924	1661	2157	438	2130	374
2162	1238	2214	160	2133	248
2465	168	2542	101	2460	610
1763	240	1800	325	1617	408
2222	180	2266	104	1786	408
2190	0	2270	0	2060	476
2074	146	1966	184	2206	367
2191	182	2282	516	2000	414
1873	387	2065	385	1930	405
2180	291	1883	217	1810	488
Mean 2130	583	2136	269	2029	400

These results therefore suggest that a diet rich in readily assimilable carbohydrate is associated with creatinuria which is quickly reduced after a change to a low carbohydrate diet.

Creatinine excretion

The daily excretion of creatinine in the 97 subjects examined varied from 1430 to 3800 mg. (see Fig. 1). Folin's observations on 30 subjects showed a variation from 1050 to 1660 mg.

The high creatinine excretion of these subjects is reflected in the "creatinine-nitrogen coefficient" [Shaffer, 1908, 2] which varies from 7.8 to 19.6 mg. creatinine-nitrogen per kg. of body weight with a mean of 11.6 (Fig. 2). Shaffer gives a range of 5.4 to 11.7 with a mean of 8 to 9 for "supposedly normal men", whilst Folin (on asylum inmates) gives a range of 3.5 to 9.8 with a mean of 6.

It is of interest to note that the subject having the highest creatinine coefficient of 19.6 could jump 6 ft. in the high jump and 12 ft. in the pole vault; in addition, his amount of daily practice was well above the average. This same individual also showed the highest daily excretion of creatine, i.e. 1200 mg.

The daily excretion of creatinine was determined in the selected groups of individuals referred to in the previous section. Neither low carbohydrate diet nor diminished amounts of exercise were accompanied by any significant change in the creatinine excretion (Tables I and II).

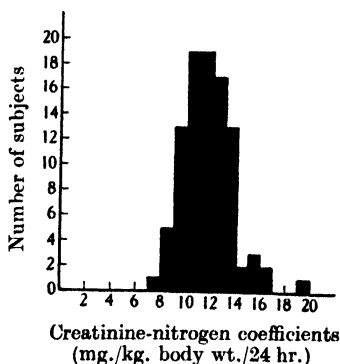


Fig. 2. Distribution of creatinine-nitrogen coefficients (mg. creatinine-nitrogen/kg. body wt./24 hr.) amongst 97 subjects.

*Variations in the output of creatine and creatinine associated with season
and increased fitness for exercise*

In order to determine if the course of physical training affected the creatinuria and high creatinine coefficients, estimations of creatine and creatinine outputs were repeated on 45 subjects of Group B during May and June 1938, i.e. towards the end of the year's training.

Although in many cases there were variations in figures for both creatine and creatinine from those obtained during October, November and December 1937, nevertheless, the high creatinine coefficients and creatinuria were maintained.

These individual variations in excretion were not all in the same direction either for creatinine or creatine, neither was any significant shift shown in the frequency distribution (Figs. 3 and 4).

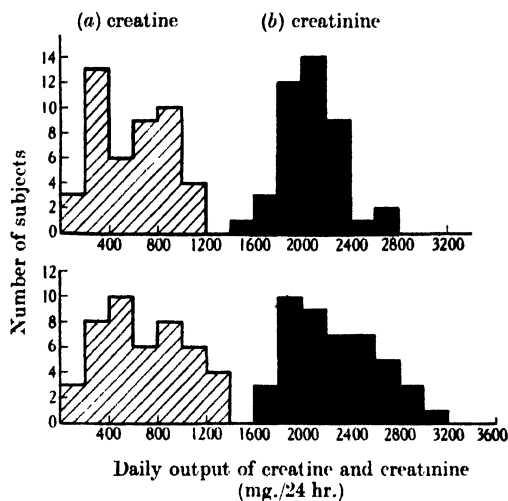


Fig. 3.

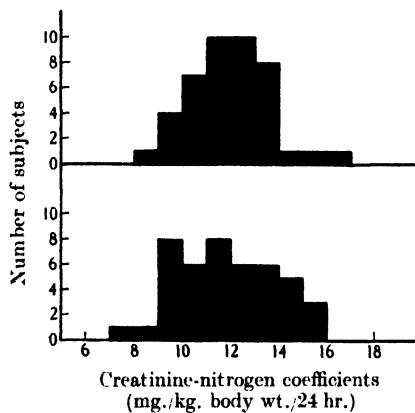


Fig. 4.

Fig. 3. (Upper): The daily output (mg./24 hr.) of creatine (a) and creatinine (b) observed in 45 subjects [Group B] in October 1937. (Lower): The same group examined in May 1938.

Fig. 4. Distribution of creatinine-nitrogen coefficients amongst the 45 members of Group B, (a) October 1937, (b) May 1938.

DISCUSSION

Creatinuria

There is evidence that muscular exercise and the ingestion of large amounts of glucose can both contribute towards the production of creatinuria, and from results described in this paper it appears that the latter factor may be the more important.

The production of creatinuria following the ingestion of glucose might be explained by experiments reported by Brentano [1932] and by Cori & Cori [1931], who found that the subcutaneous injection of insulin produced creatinuria, for the intake of glucose, especially in large amounts, presumably evokes a secretion of insulin by the pancreas.

The results presented here are in agreement with those of Haldi & Bachman [1936] who produced creatinuria by feeding glucose and fructose, but their results were obtained on only two adult subjects and the diet throughout the 24 hr. prior to and during the experiments was not indicated.

Excretion of creatinine

Since Folin [1905] suggested that the daily creatinine output might be taken as an index of endogenous metabolism, other views regarding its significance have been put forward.

Shaffer [1908, 2], Burger [1919] and McClugage *et al.* [1931] found a direct relationship between muscular development and creatinine excretion in adult men, and both Burger and Shaffer regarded the creatinine coefficient as a measure of the "participation of the muscles in the total body weight". In agreement with this view Hodgson & Lewis [1928] found that women have lower creatinine coefficients than men, with the exception that women whose muscles had been developed by muscular exercise had creatinine coefficients comparable with those of men. Talbot [1936] found in children that low coefficients were associated with obesity and high coefficients with lack of fat and well-developed muscles.

Myers & Fine [1915], working with cats, dogs and rabbits, found a relationship between the concentration of creatine in the muscles and the creatinine coefficient, and described the creatinine coefficient as being "an index of the concentration of creatine in the muscles or body". These findings have been confirmed by Palladin [1924] but have been denied by Benedict & Osterberg [1914], Chanutin [1932] and Garot [1930].

It would appear that the creatinine coefficient bears a relationship to the proportion of muscular tissue in the total body weight.

The experimental findings recorded in this paper are in agreement with these results. The subjects used in the experiment consisted of individuals picked from a large number of applicants for their ability at gymnastics and athletics. Their muscular development in proportion to total body weight was above the average and, accordingly, a high creatinine coefficient was anticipated. But the daily output of creatinine and the creatinine coefficients (creatinine-nitrogen) are higher than any hitherto recorded. The experimental findings offer no support for the idea that increased activity increases the creatinine coefficient, since there was no fall in the creatinine output in the two groups of subjects whose muscular activities were curtailed.

In view of the fact that there was no significant variation in the excretion of creatinine when subjects were placed on a low carbohydrate and high protein diet, it is not considered that the protein intake of the individuals was responsible for the high creatinine output.

Folin's original experiments were carried out on 30 inmates of an asylum, and in his paper no data are given concerning the types of patient or the nature of their muscular activity. It is more than likely that a group of such subjects taken at random for experimental purposes would contain some suffering from melancholia and probably characterized by immobility for long periods with consequent diminution in muscular development. Moreover, there is no indication of the diets or ages of these patients. Following the demonstration of the dependence of creatinine excretion upon muscular development and from a consideration of the opinions of other workers, it seems very difficult to take these figures of Folin as representative of average normal individuals, and although they are still quoted in physiological textbooks as figures for normal individuals, their revision is obviously required.

In conclusion it may be stated that an estimation of the creatinine coefficient can afford valuable evidence regarding the muscular development and obesity of the individual. Such a test should prove useful to those associated with physical

training and the assessment of the state of nutrition of individuals, including children. But the results of investigations of the excretion of creatine and creatinine should always be correlated with the diet and the amount of physical activity undertaken.

SUMMARY

1. The excretion of creatine and creatinine was investigated in 97 male students resident in a physical training college.

2. The subjects showed a higher creatinine-nitrogen coefficient (range 7.8–19.6 mg./kg./24 hr.; mean 11.6) than the accepted "normal" figure (range 5.4–11.7; mean 8–9).

3. Although creatinuria is not usually found in adult males it occurred in 96 of the 97 subjects, the range for the 24 hr. excretion being 92 to 1200 mg. with a mean of 637 mg.

4. No correlation was detected between increasing physical fitness and the magnitude of the creatine or creatinine excretion.

5. Subjects placed on a low carbohydrate diet showed a disinclination to undertake muscular work, and fatigue ensued more rapidly than usual. In these circumstances creatinuria diminished, and in some cases disappeared.

6. The probable causes of the creatinuria and the significance to be attached to the creatinine-nitrogen coefficient are discussed.

My thanks are due to Prof. A. Hemingway for his invaluable advice and criticism during the progress of this investigation; to Mr Major, Warden of the Carnegie Physical Training College, Leeds, for facilities and to the students of that college for their co-operation in the experiments.

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CLXXVI. THE V-FACTOR CONTENT AND OXYGEN CONSUMPTION OF TISSUES OF THE NORMAL AND BLACKTONGUE DOG

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It is now well established that nicotinic acid acts as a vitamin for a number of species of mammals and bacteria, and that its amide is combined in the molecule of two coenzymes which play a major part in intracellular oxidation. The question arises whether there is any connexion between these two functions. In this paper we describe experiments designed to provide information upon this point. We have determined quantitatively the V-factor which we use as a measure of the coenzyme content of the tissues in normal dogs and dogs suffering from acute deficiency of nicotinic acid ("blacktongue"), and have also measured *in vitro* the O_2 consumption of some of these tissues in the presence of suitable substrates. Our results show that the synthesis of V-factor can be restricted by greatly decreasing the intake of nicotinic acid but do not suggest that blacktongue can be explained in the simple terms of a generalized coenzyme deficiency.

The vitamin activity of nicotinic acid in curing specific deficiency diseases was first reported for the dog by Elvehjem *et al.* [1937] and extended to man by Fouts *et al.* [1937], Harris [1937] and Smith *et al.* [1937], to swine by Chick *et al.* [1938], and to the monkey by Harris [1937, 1938]. Although no specific deficiency disease has been shown to result from deprivation of dietary nicotinic acid in other species, there is evidence that it is essential for the guinea-pig [Harris, 1939] and the pigeon [Funk & Funk, 1937; Harris, 1939]. The earliest reports on micro-organisms showed that nicotinic acid is essential for the growth of *Staphylococcus aureus* [Knight, 1937] and the diphtheria bacillus [Mueller, 1937], and similar observations have since been made for the dysentery bacillus [Koser *et al.* 1938] and for *Proteus* [Fildes, 1938].

Nicotinamide was shown by Warburg to be combined in the molecule of codehydrogenases. [For brevity coenzyme I or cozymase will be designated DPN (diphosphopyridinenucleotide) and coenzyme II as TPN (triphosphopyridinenucleotide).] The precise quantitative evaluation of the parts played by DPN and TPN in cellular metabolism has not been made, but the basic importance of these substances seems to be beyond question. Their structure and definition have been reviewed by Euler [1936] and Warburg [1938], and the number, type and relationships of the systems in which they function have been discussed recently by Fischer [1939], Dixon [1939] and Martius [1939]. For our present purpose it should be recalled that the oxidation of glucose may involve either DPN or TPN, probably the latter more often. DPN is required for glycolysis resulting in lactic acid formation by muscle and probably all other tissues [cf. Meyerhof & Ohlmeyer, 1937], and for the oxidation of lactate by

kidney, heart, liver, brain, lung, diaphragm and skeletal muscle [Green & Brosteaux, 1936; Quastel & Wheatley, 1938].

A preliminary report on V-factor level in the blood of the dog has already been published by us [Kohn & Dann, 1939].

Experimental methods

Care and feeding of the dogs. The dogs were housed in metal cages having removable tray bottoms which were liberally filled with pine shavings. The trays were removed, cleaned and filled with fresh shavings every alternate day. Some of the dogs were removed for certain periods to metabolism cages exactly similar except for the replacement of the bottom tray by an openwork metal screen which allowed collection of the urine. The food and water troughs were attached to the sides of the cages in order to prevent fouling by urine or faeces. Adult dogs of both sexes and mixed breeds were used, weighing about 10 kg. each.

Two diets were used during the course of this study; these are referred to as the stock diet and the deficient diet. The stock diet consisted of horse meat, bread and cowpeas, cooked together. The horse meat included all the muscle meats of the carcass together with the bones and all other organs, except the intestines and the liver. The deficient regime was a modified Goldberger diet with certain vitamin supplements. The diet was prepared as follows: yellow cornmeal 400 parts, ground cowpeas 50 parts, Underhill & Mendel's [1928] salt mixture 12 parts and water 2000 parts were cooked for 2 hr. in an open boiler; then alcohol-extracted casein 60 parts, sucrose 32 parts and cottonseed oil 30 parts by weight were stirred into the mixture. Each dog was given 1 kg. of this food daily and its consumption was recorded. The diet was supplemented by weekly treatment with capsules of shark liver oil (40,000 International Units of vitamin A) and 1 mg. aneurin chloride orally.

Some of the dogs used in the blood study were maintained on the deficient diet until they developed blacktongue and were then cured with a small dose of nicotinamide: blood samples were taken at intervals before, during and after the attack. Others received either the deficient diet or the stock diet and were given in addition daily subcutaneous injections of nicotinic acid, either 2 mg. or 20 mg. per kg. body weight.

The dogs killed for tissue analysis fall into three groups. Those with blacktongue form Group BT. A dog was considered to be suffering from acute blacktongue when at least three of the following signs occurred together: (1) Marked reddening or necrosis of the membranes of mouth or throat. (2) Complete or almost complete loss of appetite. (3) Increased salivation. (4) Marked loss of weight. (5) Diarrhoea. The dogs were not killed until severe and unmistakable signs appeared. The normal dogs were divided into Group NC, those just cured of acute blacktongue by a small dose of nicotinamide (2 mg. per kg. body weight daily for 5 days, reckoned on the normal weight of the dog before the onset of blacktongue); and Group N, those whose regimen for some time before they were killed included liberal amounts of nicotinic acid in the diet or large amounts administered subcutaneously or both.

Solutions used for injection were two: a solution of nicotinamide in distilled water, 10 mg. per ml., sterilized by autoclaving; and a solution of nicotinic acid, 100 mg. per ml. made up by adding 4% NaOH solution to solid nicotinic acid until the pH reached 7.4, then diluting to volume with distilled water and autoclaving. The amide had been used in the beginning, but later the sodium nicotinate was substituted in the large doses for economy.

Determination of coenzyme-like substances in blood and tissue extracts. The method used for quantitative estimation of the coenzyme-like substances was that previously described [Kohn, 1938] with the slight modifications mentioned below. It is based on the discovery of Lwoff & Lwoff [1937] that either DPN or TPN can serve as the V-factor essential for the growth of *Haemophilus parainfluenzae*, whereas nicotinic acid or its amide cannot. The method does not distinguish between DPN and TPN, and it is not known whether closely related compounds such as the nicotinamide mononucleotide could serve as V-factor, or whether they occur in the body. For this reason the material assayed will be referred to as V-factor or "coenzyme-like substances". The principle of the method is to determine the quantity of extract necessary to produce an amount of bacterial growth equal to that produced by a standard. We have calibrated our standard against a DPN preparation (65% pure) kindly placed at our disposal by Prof. J. R nnstr m and Drs A. Lennerstrand and E. Sperber of Stockholm. The results are therefore expressed in equivalent amounts of DPN, or *d.e.* Thus for *blood* an assay of 10 *d.e.* signifies an activity equivalent to 10 μ g. DPN per ml. corpuscles (to which the blood V-factor is confined); for *tissues* an assay of 10 *d.e.* signifies an activity equivalent to 10 μ g. DPN per g. fresh weight of tissue.

The following modifications of the published method have been made. The broth in which the stock cultures were maintained and in which the growth tests were carried out contained in addition to the usual 2% proteose-peptone (Difco brand) and 0.6% NaCl, 0.1% sucrose and 0.04% fumaric acid, titrated with NaOH to pH 7.8 using a glass electrode. The medium was autoclaved no longer than 15 min. at 15 lb. to minimize darkening. The turbidity of the cultures was determined 20–24 hr. after inoculation. A test of the precision of the assay was made by repeating eight times over a period of 1 month a comparative assay of two different standards. The data obtained showed that the standard deviation of this series is $\pm 7\%$, so that the error of a single estimation may be expected to exceed $\pm 7\%$ once in three times and to exceed $\pm 16\%$ once in twenty times.

Preparation of blood extracts. About 0.4 ml. of blood obtained by venepuncture was diluted with 1.6 ml. of oxalated saline solution (3 ml. of 2.2% $K_2C_2O_4$, H_2O + 16 ml. 0.9% NaCl). On one occasion the diluted sample clotted, so a fresh sample was drawn and diluted with a mixture containing a larger proportion of oxalate solution. The haematocrite value of the diluted blood was determined and 0.2 ml. of the diluted blood was laked with 7.8 ml. distilled water and deproteinized by the addition of 2 ml. 2.7% trichloroacetic acid brought to about pH 3 with NaOH. The tube containing the suspension was corked and inverted to sterilize the inside walls, then stored in the cold until required. The tube was centrifuged and the clear extract assayed for V-factor.

Preparation of tissue extracts. The dog was killed by decapitation and the tissues for assay dissected out as rapidly as possible. About 50 mg. fresh weight of the tissue were weighed on a torsion balance and immediately ground with fine sand in the presence of 2 ml. 2.7% trichloroacetic acid (adjusted to pH 2.7–3.0 with NaOH) containing 2% $K_3Fe(CN)_6$. The ferricyanide converts the reduced V-factor into the oxidized form during the extraction; the oxidized form is stable but the reduced form is destroyed on standing at the pH used, therefore the ferricyanide was omitted when it was desired to measure the oxidized V-factor only. After about 30 min. a further 8 ml. of trichloroacetic acid solution without ferricyanide were added and the suspension was transferred from the mortar to a test tube, then corked, inverted to sterilize the tube, and stored in the

cold for several hours. The test tube was then centrifuged and an appropriate quantity of the clear or almost clear supernatant fluid was diluted in sterile broth until 1 ml. of solution contained the extract derived from a quantity of tissue varying from 0.25 to 1.0 mg. according to the tissue and condition of the dog. 0.1, 0.2 and 0.3 ml. of this diluted solution were added to a series of tubes, each containing 7 ml. of broth, which were then inoculated and compared.

The small quantity of tissue used, together with the relatively low pH of the extractive solution permits rapid inactivation of the enzymes which destroy DPN and TPN when the cellular structure is disrupted. In blood the addition of ferricyanide is unnecessary. The tissue extracts have been shown to be stable several days when stored at 1°.

Measurements of O₂ consumption of tissues in vitro. The O₂ uptake of the tissues *in vitro* was measured at 37.5° in the usual Warburg-Barcroft apparatus, filled with O₂. The inner cups contained alkali. After equilibration the O₂ consumption for 30 min. was determined for slices of kidney cortex and of liver, and for strips of thigh muscle, suspended in Ringer phosphate solution at pH 7.4 with 0.1 % added glucose. The Q_{O_2} (ml. O₂ per g. dry wt. per hr.) calculated was designated RG (see results in Table I). Similarly the Q_{O_2} was calculated for slices of cerebral cortex suspended in Ringer phosphate without added glucose and designated R.

After the 30th min. the side arms were tipped, adding to the control vessels 0.5 ml. of Ringer-phosphate, and to the others enough neutralized *dl*-lactate to make a final concentration of 0.024 *M*; except for brain, where enough glucose was added to make a final concentration of 0.2 %. From measurements between the 40th and 70th min. the increment in O₂ consumption due to the addition of lactate or glucose was calculated as Q_{O_2} , designated respectively L and G (see Table I).

Other measurements were made in the presence of added nicotinic acid.

Results

V-factor content of dog blood. Ten dogs were used for this phase of the study, blood samples being taken from 6 to 15 times from each dog over a total period which varied from 61 to 110 days for different animals. Some dogs were kept on the deficient diet until they developed blacktongue, then cured with small doses of nicotinamide, while others were maintained in health on the deficient ration throughout the whole period by daily administration of either a moderate or a large dose of nicotinic acid or its amide. Two dogs were kept throughout on the stock diet, with addition of large daily doses of nicotinic acid for the latter part of the period.

Wide variations in the nicotinic acid intake were found to have little effect on the V-factor content of the blood, which showed no marked change after the development of acute blacktongue. Typical results for three of the dogs are shown in Fig. 1 which indicates the variation in V-factor levels for an individual dog, and also the variation between different dogs. Altogether we have made 15 observations during 11 attacks of blacktongue and 74 observations on normal dogs. The level in the normal dogs varied from 54 to 120 *d.e.* with a mean value of 91 *d.e.* and standard deviation for the series was 15.4 *d.e.* In blacktongue dogs the level varied from 72 to 117 *d.e.* with mean 89.3 *d.e.* and standard deviation of 13.7 *d.e.* The difference between the means is not significant.

V-factor content of dog tissues. As the nicotinic acid intake varied significant changes were found in the V-factor content of liver and of striated muscle

(combined results on diaphragm and thigh) but not of heart, pancreas, kidney or brain. The data are compiled in Table I, which gives individual readings on various tissues from 19 dogs, together with mean values respectively for dogs with blacktongue (Group BT), normal dogs just after cure of blacktongue by small doses (Group NC) and normal dogs on a liberal regimen of nicotinic acid

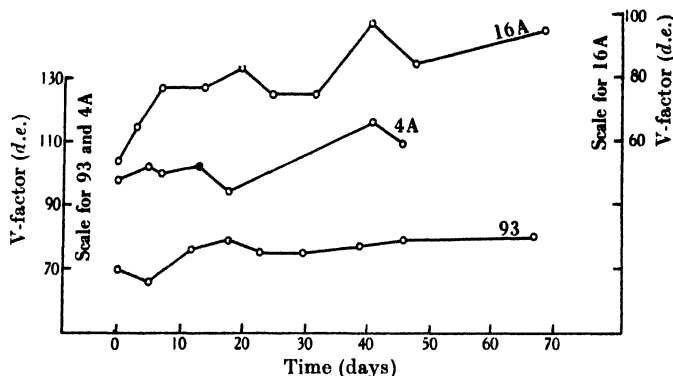


Fig. 1. Variation in the blood level of V-factor. Dog 4A after a period on the deficient diet developed acute blacktongue on the 1st day recorded and was given 2 mg. nicotinamide per kg. subcutaneously each of the first 5 days. It was cured by the 7th day and relapsed into severe blacktongue again on the 45th day. Dog 93 received the deficient diet + 2 mg. nicotinamide per kg. daily for 18 days before the first reading. This regimen remained unchanged until the 25th day, when the daily dose of nicotinamide was increased to 20 mg. per kg. until the 55th day, after which the nicotinamide was discontinued and 10 ml. crude aqueous liver extract (=300 g. liver) were given daily by mouth.

Dog 16A is included because it was the only dog which showed a marked and sustained change in V-factor level. It was an emaciated but otherwise normal adult dog brought to the laboratory and placed on stock diet 5 days before the first reading. Until the 27th day the diet was unsupplemented, and afterwards 20 mg. nicotinamide per kg. were injected daily until the end. During the period of observation its weight increased from 15 to 19 kg.

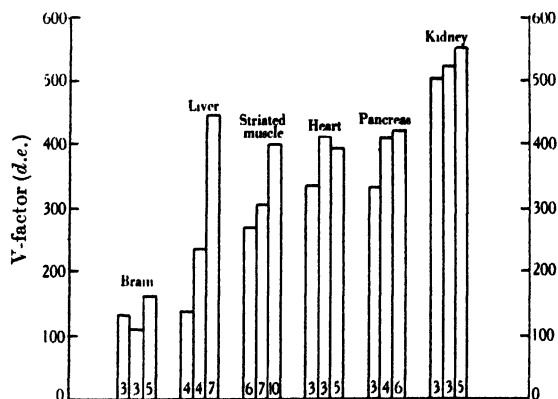


Fig. 2. Mean V-factor content of tissues from blacktongue and normal dogs. For each tissue the column at the left is for Group BT, middle for Group NC and right for Group N. The figure at the base of each column gives the number of observations from which the mean was calculated.

(whether on stock diet supplemented by nicotinic acid or amide, or on stock diet with or without supplement) (Group N). They show the wide variability of total V-factor content of most of the tissues for dogs within any one group and similar variability in the proportion of V-factor present in the oxidized form.

Table I

Data on the V-factor content and *in vitro* oxygen consumption of tissues from 19 dogs. Blank spaces indicate that no measurement was made on the appropriate tissue; no readings have been omitted. Dogs in Group BT were killed after development of severe blacktongue on the deficient diet. Dogs in Group NC were killed after cure of blacktongue by administration of an adequate but not excessive amount of nicotinic acid. Dogs in Group C were killed after receiving liberal or large amounts of nicotinic acid. A, B and C had been fed the stock diet for at least 1 month. No. 69 received the stock diet for 3 months and then the deficient diet for 3 weeks. No. 89 received stock diet + 20 mg. nicotinic acid per kg. for 25 days. No. 100 received deficient diet + 2 mg. per kg. for 40 days, then deficient diet + 20 mg. per kg. for 20 days. No. 25 A received deficient diet + 20 mg. per kg. for 12 days.

Amounts of V-factor are expressed in d.e. (see text).

Group	Dog no.	Liver				Diaphragm		Thigh				Heart	
		V-factor d.e.		Q _{O₂}		V-factor d.e.		V-factor d.e.		Q _{O₂}		Total Oxidized	
		Total	Oxidized	RG	L	Total	Oxidized	Total	Oxidized	RG	L	Total	Oxidized
Group BT	32 A	165	< 90	—	—	—	—	160	< 80	—	—	—	—
Dogs killed during an attack of blacktongue	4 A	90	—	—	—	—	—	385	100	—	—	355	180
	98	100	—	9.5	1.4	290	< 80	190	< 80	2.6	0	345	150
	18 A	180	< 60	6.4	0.3	190	100	400	250	2.6	0.1	295	105
	12 A	—	155	8.9	0	—	265	—	—	—	—	—	305
	14 A	—	170	10.2	1.1	—	—	415	2.8	0.4	—	—	324
	13 A	—	165	7.3	1.2	—	275	—	—	5.2	0.6	—	260
Group NC	80	285	< 140	—	—	—	—	290	120	—	—	—	—
Normal dogs killed just after cure of blacktongue	24 A	210	< 80	5.2	1.4	345	110	240	185	3.8	0.4	270	255
	7 A	315	158	5.5	1.8	275	145	395	265	2.7	0.5	585	420
	3 A	125	35	6.0	1.3	205	95	380	295	3.8	0.4	380	315
Group N	89	355	< 170	—	—	—	—	550	320	—	—	—	—
Other normal dogs, see below	C	460	< 150	—	—	165	—	335	285	—	—	335	135
	26 A	425	305	6.6	1.6	445	370	360	230	5.2	0.6	420	305
	100	370	170	6.4	0.9	—	—	495	315	4.2	0.6	410	75
	B	655	380	6.9	0.6	425	405	375	—	4.7	1.1	450	355
	25 A	385	—	—	—	420	255	420	—	—	—	345	240
	69	—	225	6.0	1.3	—	—	405	4.3	0.5	—	—	365
	A	463	273	6.3	1.9	—	—	580	4.4	0.5	—	—	355
Mean of Group BT		134	106	8.5	0.8	240	180	284	175	3.3	0.3	332	220
Mean of Group NC		234	97	5.6	1.5	275	117	326	208	3.4	0.4	412	330
Mean of Group N		445	235	6.4	1.4	364	270	422	363	4.5	0.6	392	296

Group	Dog no.	Pancreas		Kidney				Brain					
		V-factor d.e.		V-factor d.e.		Q _{O₂}		V-factor d.e.		Q _{O₂}			
		Total	Oxidized	Total	Oxidized	RG	L	Total	Oxidized	R	G	L	
Group BT	32 A	475	205	—	—	—	—	—	—	—	—	—	—
Dogs killed during an attack of blacktongue	4 A	—	—	660	520	—	—	140	110	—	—	—	—
	98	260	160	490	315	14.8	2.1	115	95	7.7	2.0	3.0	—
	18 A	270	280	360	235	15.6	1.2	135	90	7.0	3.1	3.8	—
	12 A	—	—	—	340	15.3	1.8	—	105	10.2	3.1	4.6	—
	14 A	—	320	—	295	18.1	2.6	—	220	8.7	4.4	5.5	—
	13 A	—	295	—	265	18.4	2.9	—	155	8.2	2.3	2.9	—
Group NC	80	475	425	—	—	—	—	—	—	—	—	—	—
Normal dogs killed just after cure of blacktongue	24 A	485	410	520	270	15.3	3.9	85	85	8.8	2.1	4.2	—
	7 A	360	320	450	340	—	—	104	103	7.9	3.5	4.6	—
	3 A	320	—	600	345	15.9	2.7	135	135	8.2	3.7	4.7	—
Group N	89	410	390	—	—	—	—	—	—	—	—	—	—
Other normal dogs, see below	C	430	345	420	350	—	—	155	140	—	—	—	—
	26 A	445	390	730	630	14.9	3.9	160	95	8.0	3.7	4.9	—
	100	500	380	545	390	15.8	6.2	165	140	9.8	3.0	0.3	—
	B	340	295	510	265	15.2	4.6	215	205	—	—	—	—
	25 A	400	345	555	290	—	—	105	65	—	—	—	—
	69	—	275	—	255	14.8	3.3	—	181	—	—	—	—
	A	—	275	—	310	16.8	1.9	—	167	—	—	—	—
Mean of Group BT		335	252	503	328	16.4	2.1	130	129	8.4	3.0	4.0	—
Mean of Group NC		410	387	523	318	15.6	3.3	108	107	8.3	3.1	4.5	—
Mean of Group N		421	337	552	355	15.5	4.4	160	142	8.9	3.4	3.8	—

The tissue in which least variability was found is the heart muscle, which in five dogs of Group N contained from 335 to 450 d.e.

The data also show the upward trend of the mean V-factor content for the various tissues from Group BT to Group NC and Group N (see Fig. 2), but this must be interpreted with caution on account of the variation within individual groups. The means in each column of Table I have been compared by statistical analysis of the individual figures in each group, and application of the *t*-test of significance [Fisher, 1936], using the 5 % point as the level of significance (see Table II).

Table II. *Significant differences between Group N and Groups NC and BT*

Tissue	Group	Total V	Respiration
Liver	BT	0.30	1.35 (RG)
	NC	0.55	—
Muscle	BT	0.65	0.75 (RG)*
	NC	0.75*	0.75 (RG)
Kidney	BT	—	0.50 (L)
	NC	—	—

The differences are calculated by comparing the group means at the foot of Table I; Groups BT and NC are expressed as ratios to Group N and each figure is given to the nearest 0.05. A blank indicates that the difference observed was not significant (see text). An asterisk indicates that the difference fell just short of significance, but the presence of a single very discrepant figure in one of the groups compared suggested that additional data would show the difference to be significant.

The figures for V-content of muscle are the combined data of Table I on diaphragm and thigh muscle, but respiration measurements were made only on thigh.

A number of observations which will not be reported in detail showed that in organs excised immediately after the dog was killed about 20 % of the V-factor was destroyed on standing for 1 hr. at room temperature (25°).

The O₂ consumption of tissues in vitro. Changes in the O₂ consumption were observed in those tissues in which the V-factor changed (liver and muscle) and also in kidney, where the V-factor remained steady. These data are shown in Table I. Again all significant differences between means of different groups are collected in Table II. In addition, some observations on the effect of nicotinic acid on O₂ consumption were made. In the presence of 1.3×10^{-3} to 2.7×10^{-3} M nicotinic acid at pH 7.4 the RG value for kidney was depressed about 11 % but the increment in uptake produced by lactate was increased about 65 %.

Table III. *Relative amounts of V-factor in various tissues compared with the relative rates of oxygen consumption in vitro and in vivo*

Tissue	V-factor	Respiration	
		<i>In vitro</i>	<i>In vivo</i>
Muscle	1	1	1
Liver	1.1	1.4	1.5
Brain	0.4	2.7	3.0
Pancreas	1.0	—	5.0
Heart	0.95	—	10.5
Kidney	1.3	3.4	10.5

Striated muscle is used as the standard of comparison. *In vitro* respiration was calculated from the data of Table I, using RG for liver, thigh and kidney, and R + G for brain. *In vivo* respiration during maximal activity for brain is based on the reviews of Page [1937] and Quastel [1939]; for other tissues on figures given by Loewy [1925].

The tissues used were obtained from one blacktongue and four normal dogs. No effect was observed with liver or with muscle.

In Table III are given the relative O_2 consumptions of the tissues of normal dogs compared with the relative V-factor contents and with the respiration rates *in vivo* during maximal activity as given by various reviewers. The rate of O_2 consumption per unit amount of V-factor is seen to vary greatly in different tissues. Thus *in vivo* it is from six to ten times greater for heart, brain and kidney than for muscle and liver. *In vitro* brain consumes far more than kidney, muscle and liver. These data show that the relationship between V-factor and O_2 consumption is not simple.

Note on the dry weight of tissues in blacktongue. Since the terminal stages of acute blacktongue are accompanied by dehydration, it appeared possible that our assays of V-factor on tissues of blacktongue dogs might yield figures too high for true comparison with normals on account of loss of water from these tissues. To test this possibility the percentage dry weight of tissues was determined for one dog in each group by drying small samples to constant weight at 110° . The figures are given in Table IV and indicate that there was no increase in the percentage of solids in these tissues in blacktongue.

Table IV. *Dry weight as percentage of fresh weight*

Dog no.	Group	Liver	Kidney	Heart	Pancreas	Brain	Diaphragm	Thigh
18 A	BT	27	22	24	24	—	25	23
24 A	N ⁽¹⁾	30	24	21	24	19	—	25
26 A	N	31	22	23	33	20	27	—

DISCUSSION

The interpretation of the results given above depends upon the specificity of the V-factor assay. It has already been mentioned that V-factor activity is possessed only by DPN and TPN among known compounds, but there is a possibility that closely related compounds which exert this activity occur unrecognized in tissues. Our mean figures, however, for normal dogs are of the same order as figures reported in the literature for DPN content. These figures, expressed in $\mu\text{g. per g.}$ fresh tissue, are as follows: rabbit skeletal muscle, 400 [Ochoa & Ochoa, 1937]; beef muscle 310, beef liver 700, pig liver 520 [Axelrod & Elvehjem, 1939]; rat muscle, heart, kidney and liver 150–215 [Euler *et al.* 1938]. The percentage of coenzyme in the reduced form varies from 25 to 50 [Ochoa & Ochoa, 1937; Euler & Heiwinkel, 1937; Euler *et al.* 1938]. In rat tissues the amount of TPN is said to be from 15 to 50 % of the amount of DPN [Euler *et al.* 1938]. The older data cannot be reduced to absolute values or else the authors ignore the reduced forms. In view of the lack of evidence to the contrary, and in consideration of the foregoing, we shall assume that the V-factor content is a direct index of the coenzyme content of tissues, either because DPN and TPN are alone in possessing V-factor activity, or because they occur in tissues together with closely related compounds in fairly constant proportions.

Our results as summarized in Table II show that the tissues of the dog may be divided into two classes, those whose V-factor level is influenced by the intake of nicotinic acid, and those whose level is not. To the former belong striated muscle and, particularly, liver. In blacktongue, striated muscle was down to 65 % and liver to 30 % of the value for Group N. When a cure was obtained with an adequate but not excessive dose of nicotinic acid, the fractions rose only to 75 and 55 % respectively and these values were compatible with normal health.

The stability of the V-factor level in brain, kidney, pancreas and blood indicates that some factor other than nicotinic acid intake controls the levels in these tissues. Possibly they are maintained at the expense of liver and striated muscle. The steady rise in the level for dog 16A (Fig. 1) does not contradict this conclusion. When first brought into the laboratory, the animal was quite emaciated, but showed no signs of blacktongue. For this reason, the subsequent increase in weight accompanied by an increase in blood level should probably be attributed to a lack of some "building stone" other than nicotinic acid.

In man, on the other hand, the administration of nicotinic acid causes a considerable rise in the blood level; in pellagra the latter is somewhat depressed, but on account of the large variations among healthy subjects it remains within normal limits. Thus both in man and the dog, but for quite different reasons, the V-factor level of the blood is of no diagnostic value.

The only observations in the literature are contained in the preliminary statement of Axelrod & Elvehjem [1939]. They compared one deficient dog with normals, and without supplying the data stated that the DPN content of liver decreased by 100 % and of muscle by 1000 % (*sic*). We have found liver to decline more than muscle, a difference which might be attributed to the larger size of our sample or, alternatively, to a much greater decrease in DPN than in V-factor. Their statement that "the cozymase content of the tissues of normal animals remains quite constant" is definitely contrary to our results with liver and muscle.

Only a few data are to be found in the literature on the proportion of tissue coenzymes present in the reduced form (see citations above), and these are for normal animals only. Our observations show variations from no reduced coenzyme-like substance in the brain of each of three dogs of Group NC to a mean of 57 % in the heart muscle of three dogs with blacktongue. There is suggestive evidence that the proportion of reduced coenzyme-like substances is increased with onset of blacktongue in all the tissues examined except heart and kidney where it remains unchanged, and liver for which no comparative figures were obtained.

The changes in V-factor content and O_2 consumption *in vitro* do not run parallel, as shown in Table III. In liver, a 70 % decrease in V-factor was accompanied by a 35 % increase in RG; in striated muscle, a 35 % decrease was accompanied by a 25 % decrease in RG; in kidney, no change in V-factor was accompanied by a 50 % decrease in L. It should be noted that in striated muscle the changes are approximately the same in the BT and NC groups, although the disease exists only in the former. Their presence therefore does not cause the signs of blacktongue.

The relation of these phenomena to the onset of blacktongue and the ultimate death of the dog is obscure, especially since no striking pathological changes peculiar to the disease itself have been described. In acute blacktongue the mucous membranes of the mouth and alimentary tract are invaded by fusospirochetal organs [Smith *et al.* 1937], the dog ceases to eat, and later to drink. There is anuria and often severe diarrhoea and dehydration. These signs, however, are of a secondary nature, and it is to be emphasized that a typical blacktongue syndrome can be produced by a deficiency of vitamin A [Underhill & Mendel, 1928; Smith *et al.* 1937].

The situation may be summarized as follows. The experimental evidence argues against the explanation of blacktongue in the simple terms of a general deficiency of DPN and TPN accompanied by a generalized inability to carry out cellular oxidation-reduction reactions as has been suggested by Euler *et al.* [1939].

On the other hand, the level of these coenzymes is definitely reduced in striated muscle and in liver, and changes in the oxidative metabolism of liver, muscle and kidney can be detected. Of the several possibilities for explanation which suggest themselves, we believe the two following deserve special mention.

(1) When the V-factor level of the liver is reduced below a certain limit, some reaction upon which the maintenance of health depends is impaired because sufficient coenzyme is not available.

(2) Nicotinic acid enters the metabolism in essential ways as yet unknown.

The second possibility has been suggested previously [Daft *et al.* 1938; Kohn, 1938] and has been discussed by Dann [1939], but the evidence is inconclusive, and there is no way at present of favouring either.

It is of interest that these results contrast sharply with those reported for aneurin deficiency where the level of aneurin falls very markedly in brain, heart, muscle and liver, and is accompanied by a decrease in the activity mediated by the coenzyme formed from the vitamin [Ochoa & Peters, 1938; Banga *et al.* 1939; Lu, 1939].

SUMMARY

1. The concentration of coenzyme-like substances (V-factor) has been determined in the brain, heart, striated muscle, liver, kidney, pancreas and blood of dogs (a) with acute blacktongue due to nicotinic acid deficiency, (b) in normal health immediately after cure of blacktongue by small doses of nicotinic acid and (c) in normal health after receiving liberal or large amounts of nicotinic acid. The O_2 consumption of brain, liver, kidney and striated muscle was studied *in vitro*.

2. In dogs with acute blacktongue the concentration of coenzyme-like substances was 70 % lower in the liver and 35 % lower in striated muscle, compared with dogs receiving liberal or large amounts of nicotinic acid. In dogs just cured of blacktongue liver and muscle were 45 and 25 % lower respectively. No difference was found in the other tissues examined.

3. The decreased coenzyme content of the liver in blacktongue was accompanied by an increase of 35 % in O_2 consumption; in kidney the coenzyme content was unchanged but ability to oxidize lactate was decreased by 50 %.

4. In blacktongue the percentage of coenzyme in the reduced form is increased in most tissues except brain and kidney.

5. No evidence was found indicating that blacktongue results from a generalized decrease of coenzyme level in the tissues.

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CLXXVII. THE PECTIC SUBSTANCES OF PLANTS

VI. THE RELATION BETWEEN JELLY STRENGTH, VISCOSITY AND COMPOSITION OF VARIOUS PECTINS

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THE conditions of formation of pectin-sugar-acid jellies have been exhaustively studied in recent times, notably by Tarr [1923; 1926] and Tarr & Baker [1924] in America, and by Ogg [1924] in Great Britain, and the principles laid down have been extended in lengthy investigations by Myers & Baker [1926-34]. In spite, however, of a mass of data obtained and analysed by the latter workers with reference to the relation between composition and jelly strength, the problem is still an open one.

Many workers have insisted that an adequate methoxyl content is a prerequisite of jelly formation by a pectin and that methoxyl content may be used as a criterion of jelly strength. These beliefs are denied by Myers & Baker who find that methoxyl content is not a measure of jelly strength: the power of forming jellies may be completely destroyed without loss of methoxyl and conversely the methoxyl content may be considerably lowered without appreciable loss of jelly strength.

Myers & Baker found that no single constituent of the pectin complex was a criterion of jelly power. Recent views on pectin constitution have replaced those on which they based their arguments, but they made the important suggestion that jelly power was a function of the degree of polymerization of the galacturonic acid. The work of Schneider & Henglein [1936], Schneider & Bock [1937] and Schneider & Fritschi [1937], Hirst & Jones [1938; 1939], Beaven & Jones [1939] and of Smith [1939] indicates that the essential part of the pectin complex consists of a long chain of galacturonic acid units, the araban and galactan usually found in most preparations being regarded as adsorbed non-essential constituents. Since viscosity is a measure of molecular size and since viscosity and jelly strength are related, the suggestion of Myers & Baker may be restated in the form that jelly strength is a function of molecular size, or of chain length of the essential constituent.

In the present investigation an attempt has been made to correlate jelly strength with viscosity, and chemical composition with both, in the case of pectins prepared from different sources by different methods. Artificial esters produced by methylation of pectic products have also been employed in the course of the study. The views of Myers & Baker, who used pectin from one source, prepared by the same method throughout, have been largely confirmed for the more general case indicated.

EXPERIMENTAL

Preparation of materials

Pectolic acid. This product was originally prepared by Ehrlich & Schubert [1929] and by Ehrlich & Guttman [1933] and their methods were employed in this instance with but slight modification.

50 g. of commercial citrus pectin were gradually added with rapid mechanical stirring to 750 ml. of 5% HCl in a bolt-head flask maintained at 70–75°. After heating for 1½ hr. precipitation of the flocculent voluminous pectolic acid was complete. The precipitate was filtered off when cool, washed first with 2% HCl and then with water to the point of solution. After boiling with 95% alcohol for 30 min., the precipitate was air-dried. Purification was effected by dissolving 20 g. in 2 l. of boiling water, cooling in the refrigerator and adding 120 ml. of conc. HCl. The precipitate was filtered off, washed in dilute HCl, then water and finally 50% alcohol until all trace of acid was removed. It was dried in graded strengths of alcohol, then *in vacuo* and finally in a Fischer drier at 78° using P₂O₅. The final product was a white powder, free from ash, and contained 94.24% anhydrogalacturonide.

Methyl pectolate and pectate. Buston & Nanji [1932] described a method for the preparation of methyl esters of pectic acid by treating the Ag salt with CH₃I under pressure. The elimination of the colloidal AgI formed is a difficulty which these workers overcame by warming with a little KBr. In preliminary experiments we found that satisfactory coagulation of the AgI was still difficult and other means of separation were sought, including saturation with quinine hydrochloride, or filtration through kieselguhr. No modification was entirely satisfactory but it was found that a larger excess of CH₃I produced the AgI in a form which could be readily filtered or centrifuged.

The method finally adopted was as follows: 2 g. of Ag pectate or pectolate were heated at 90° for 16 hr. in a pressure bottle with 25 g. CH₃I in 30 ml. CH₃OH. The mixture was then poured into 250–300 ml. of water and heated to 97° for a few minutes. The liquid was allowed to cool and filtered through paper pulp; if the filtrate was not clear at this stage it was centrifuged. The clear filtrate was precipitated with alcohol, the slimy precipitate being separated and dried as usual.

Pectins. Pectins were prepared from orange peel, apple pomace and beet pulp by methods which have been described in previous communications (see especially, Norris & Resch [1937]). Samples were prepared from these sources by: (a) ammonium oxalate extraction [Norris & Schryver, 1925]; (b) autoclave extraction [Ehrlich, 1932; Ogg, 1934]; (b 20 and b 40) autoclave extraction in presence of 20 and 40% sucrose; (c) H₂SO₄ (N/10) extraction [Food Manufacture, 1936]; (d) HCl extraction at pH 1.4 and 60° [Myers & Baker, 1929; 1931]; (d 40) HCl extraction at pH 1.4 and 60° in presence of 40% sucrose.

These methods do not require further comment at this point.

Experimental methods

Determination of jelly strength. A slightly modified form of the pectinometer described by Buston & Nanji [1932] was used. It consisted essentially of a pan on to which weights could be loaded, connected over pulley wheels to a plunger in a cylinder which contained the jelly. The movement of the plunger through the jelly with steadily increasing weights in the pan was indicated by a pointer which magnified the movement considerably. All parts coming into contact with the jelly were made of stainless steel.

A standard procedure for preparing the jellies was adopted: the requisite weight of pectin (0.10 or 0.20 g.) was dissolved in 10 ml. of 0.2% tartaric acid with 6 g. of sucrose in a 30 ml. beaker. The mixture was evaporated to 10 g. by boiling, and the hot liquid poured into the container of the pectinometer and allowed to set at 13°. Preliminary experiments had shown that jelly strength was constant after 20 hr., and 20–24 hr. were allowed for setting before taking strength readings. The strength is simply recorded as the weight in the pan required to give a predetermined definite movement of the plunger in the jelly.

Determination of viscosity. The viscosity was determined by means of Ostwald type viscometers maintained at 25° in a constant temperature water bath. In all cases a 0.5% solution (on a moisture-free basis) of the product was used.

Analysis of products. The analytical methods adopted were as given by Norris & Resch [1937]. It may be pointed out, however, that the results given for "anhydrogalactose" are difference figures and pending actual determination of galactose must be treated with reserve.

Jelly strength of methyl esters of pectolic and pectic acids

Short of complete degradation to galacturonic acid, pectolic acid appears to be the simplest derivative of pectin. Since a number of workers have suggested that methoxyl groups are a desirable, if not essential requirement for jelly formation, the jelly strength of methyl pectolate was investigated. A typical sample had the composition: uronic anhydride, 85.64%; methoxyl, 13.37%; but in no case did such a sample yield a jelly.

The methyl esters of pectic acids were next investigated. The products from beet gave no jelly even in cases where the product contained as much as 11.7% methoxyl. In one case only of samples from orange peel was a jelly obtained and this was of very poor strength, not comparable with that of orange peel pectins. Again, with the ester of pectic acid derived from commercial citrus pectin of high jelly strength, no samples were obtained which gave jellies. This in spite of the fact that the esterification was carried out under the most gentle conditions, and all details of preparation were arranged in accordance with what experience had indicated to be the best way to ensure maximum jelly strength in the product. For comparison, the analysis of a typical pectin ester and the commercial pectin from which it was obtained via the pectic acid, are given:

	Uronic %	Anhydro- galactose %	Anhydro- arabinose %	Methoxyl %	Ash %	Jelly strength g. (1% jelly)
Ester	84.60	1.28	1.04	13.08	0.33	0
Pectin	86.56	4.14	0.91	8.39	3.14	207

It is thus obvious that methoxyl content plays no essential part in jelly formation. However, a certain degree of esterification is necessary if the product is to be readily soluble in water, and in this limited sense methoxyl is a requisite of jelly formation.

As a result of esterification there is no radical change in the composition of the products but some change must occur which prevents jelly formation. This may possibly be a resolution of the complex into components which are then co-precipitated by alcohol, or, more probably, a disaggregation of the polygalacturonide into smaller units.

Jelly strength of pectins

As a preliminary to jelly strength tests on prepared pectins, the effect of the drying treatment was first examined. A number of samples of the same commercial citrus pectin were dried for varying periods in an oven at 98° and the jelly strength then determined for 1 % jellies, moisture content being allowed for. The results are shown in Fig. 1.

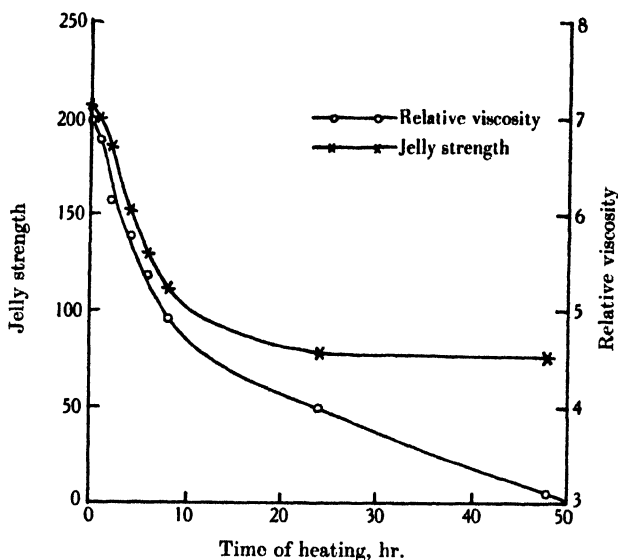


Fig. 1.

"Relative viscosity" is discussed later. After several days' heating, no jelly could be obtained.

There is thus a very serious loss in jelly strength on heating. Drying at 78° in a Fischer drier for 48 hr. involved no measurable loss in jelly strength, but in order to obviate any possible source of error heat-drying was abandoned, the products being dried in a vacuum desiccator at room temperature.

Table I shows the jelly strength of prepared pectins in relation to their source and mode of preparation. In this and subsequent tables jelly strength is given as g. for a 2% jelly.

Table I

Method of preparation	Orange peel		Apple	Beet
	I	II		
Ammonium oxalate (a)	230	122	117	Nil
Autoclave (b)	Nil	Nil	Nil	—
Autoclave + 20 % sucrose (b 20)	—	Nil	Nil	—
Autoclave + 40 % sucrose (b 40)	—	Nil	Nil	—
H ₂ SO ₄ (c)	—	—	—	Nil
HCl; pH 1.4; 60° (d)	280	330	107	Nil
HCl; pH 1.4; 60° + 40 % sucrose (d 40)	—	315	—	—

Remarkably few of the pectins obtained yielded jellies at 2% concentration, and it may be noted that since it is unusual for investigators of pectin to determine the jelly power of their products, it is probable that in many instances such products did not possess the essential jelling characteristic of pectin.

Two facts are at once observable from the table: first, no beet pectin gave a jelly, a fact fairly generally recognized. It is of interest to point out that Ehrlich [1932] used "pectins" from beet in his researches. The only claim to the production of jellies from beet pectin of which the authors are aware is contained in an article [*Food Manufacture*, 1936] which mentions jellies from beet pectin prepared by 40 hr. extraction at 60° with $N/10$ H_2SO_4 .

Secondly, no pectin prepared by autoclave extraction would yield a jelly. Tarr [1923; 1926] had found that boiling a pectin in presence of acid decreased the jelly strength, but that if sucrose were present the boiling period could be considerably prolonged without any deleterious effect. It was thought that a similar protective effect might be exhibited during the extraction of pectins by autoclaving or with acid; hence the use of 20 and 40% sucrose in some of our preparations. The addition of these large amounts of sucrose had no harmful effect on the extraction and preparation but did not prevent loss of jelly power of the products.

Table II

Source	Method of preparation	Urone %	Anhydro-arabinose %	Anhydro-galactose %	Methoxyl %	Jelly strength g.
Orange peel II	(d)	75.52	4.89	7.23	12.36	330
Orange peel II	(d 40)	78.92	4.27	6.04	10.77	315
Orange peel I	(d)	73.20	7.76	10.07	8.97	280
Orange peel I	(a)	69.32	13.87	8.57	8.24	230
Orange peel II	(a)	76.64	9.09	5.56	8.71	122
Apple	(a)	70.72	9.71	9.73	9.84	117
Apple	(d)	67.72	5.39	16.83	10.06	107
Beet	(d)	56.12	17.44	17.79	8.47	Nil
Beet	(d)	50.36	15.38	26.38	7.88	Nil
Beet	(a)	59.24	21.60	9.55	9.61	Nil
Beet	(a)	59.16	16.45	14.89	9.50	Nil
Beet	(a)	57.28	22.69	12.89	7.24	Nil
Beet	(a)	51.84	25.02	15.69	7.45	Nil
Beet	(c)	66.04	9.19	18.54	6.18	Nil
Orange peel I	(b)	60.12	14.23	16.81	8.84	Nil
Orange peel II	(b)	72.12	8.87	7.42	11.59	Nil
Orange peel II	(b 20)	70.56	13.68	7.18	8.58	Nil
Orange peel II	(b 40)	74.52	9.39	4.63	11.46	Nil
Apple	(b)	64.54	6.15	19.32	9.99	Nil
Apple	(b 20)	62.27	6.92	20.85	9.96	Nil
Apple	(b 40)	62.73	6.78	20.64	9.85	Nil

Table II shows the complete analysis of products, from which it may be observed that the relation between chemical composition and jelly power is vague at best. No definite generalizations can be made, but certain points of interest may be indicated.

There is no direct relation between urone content and jelly strength. With the exception, however, of orange peel II pectins prepared by methods (b), (b 20) and (b 40) which are anomalous throughout, it will be seen that a high urone content favours jelly formation and conversely a high content of non-urone constituents—arabinose and galactose—inhibits jelly formation. Thus, apart from the anomalies mentioned, no pectin with less than about 68% urone gave a jelly. In considering chemical composition and jelly formation it may be mentioned that the most satisfactory sample in regard to jelly strength was a commercial sample of citrus pectin which had a urone content of 86.56%, an arabinose content of less than 1% and gave a jelly strength of 207 g. for a 1% jelly.

Reference to the methoxyl contents shows that there is no relation between jelly power and methoxyl content as previously indicated.

Viscosity relationships

Myers & Baker [1926-34] have shown that jelly strength of a pectin is a function of the viscosity of the pectin solution from which the jelly is made, provided that the pectins are derived from the same source by the same method of preparation.

In what follows an attempt has been made to correlate jelly strength and viscosity, and viscosity and chemical composition for pectins from different sources prepared by different methods.

Preliminary observations revealed the important phenomenon that the viscosity of the solution decreased for some hours after its preparation. Owing to the time necessary to effect solution it was impossible to take readings earlier than 1 hr. after addition of the pectin to the water, but thereafter readings were taken at intervals as shown in Table III.

Table III

Time of standing hr.	Relative viscosity
1	7.46
2.25	7.15
3	7.07
5	6.98
26.33	6.83

A similar effect was observed with all pectins examined. Thus, for comparable viscosity readings the time of standing must be standardized, and in all subsequent measurements this time was fixed at 5 hr.

It has been shown previously that whilst heat treatment has a deleterious effect on jelly strength, no change in composition is observable. Heat treatment thus affords a simple method whereby changes in jelly strength and viscosity can be measured for the same pectin. Results of this type have been shown in Fig. 1.

The graph showing change in viscosity with time of heating is similar to that showing changes in jelly strength. It may be interpreted possibly as showing the result of hydrolytic cleavage of the pectin in the presence of a small amount of moisture. The moisture content was 4.01 % after 1 hr. and 1.10 % after 8 hr.

When viscosity is plotted against jelly strength a curve (Fig. 2) is obtained whose points, within the optimum range of the pectinometer (80-200 g.), lie in a straight line, indicating strict proportionality. The divergence of the curve from the straight line outside such range is due to experimental difficulties in measuring the strength of a very stiff jelly on the one hand and of a very syrupy jelly on the other.

Thus, proportionality between jelly strength and viscosity may hold for one particular pectin submitted to varying degrees of heat treatment, but it is by no means certain that the same holds for different pectins prepared by different methods. This is exemplified in Table IV.

Inspection of the first two columns of the table shows that there is no direct relationship but that in general there is a certain parallelism. The differences are largely due to wide variations in composition and previous history. It would seem that there is a limiting viscosity below which jelly formation does not take place, but at the same time the viscosity may be above this limit and the pectin still fail to give a jelly. An anomaly of this type is exemplified by the non-jellying pectin having a relative viscosity of 3.16: this was a beet pectin prepared

by HCl extraction at pH 1.4 and 60° . Two explanations of this may be offered: (a) that in beet pectin the polyuronide molecule is not large enough for jelly

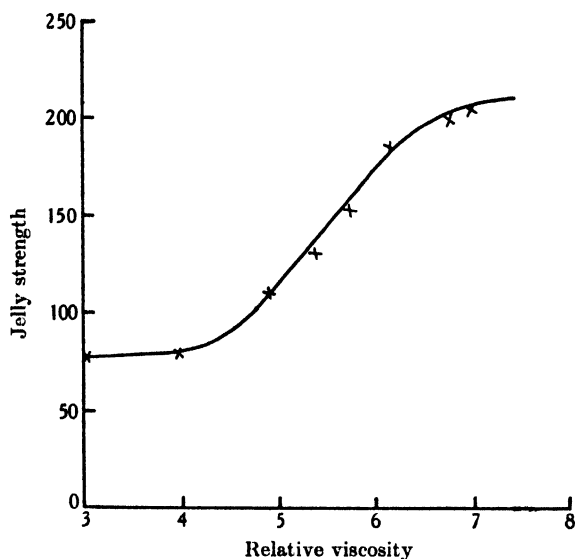


Fig. 2.

Table IV

Relative viscosity	Jelly strength 2% pectin g.	Relative viscosity of non-jellying pectins
5.20	330	3.16
3.58	315	1.64
2.38	122	1.62
2.10	107	1.42
2.09	65	1.38
1.94	117	1.36
		1.35

formation and it is the other substances present which give the pectin as a whole the viscosity observed; (b) it is more likely, however, that although the polyuronide molecule is of sufficient size, as indicated by viscosity, the contaminating substances are present in sufficient quantity to prevent jelly formation. This pectin contained over 40 % of non-urone substances.

The relationship between viscosity and source and method of preparation of pectins is indicated in Table V.

Table V

Method of preparation	Orange peel II	Apple	Beet
Ammonium oxalate (a)	2.38	1.94	1.64
Autoclave (b)	1.42	1.35	—
Autoclave + 20 % sucrose (b 20)	—	1.38	—
Autoclave + 40 % sucrose (b 40)	1.62	1.36	—
HCl; pH 1.4; 60° (d)	5.20	2.10	3.16
HCl; pH 1.4; 60° + 40 % sucrose (d 40)	3.58	—	—

This table yields, for relative viscosity, similar information to that of Table I for jelly strength. In addition, viscosity provides a measure for those

solutions which failed to give a jelly. The highest viscosity obtainable by auto-claved preparations was 1.62, the majority averaging about 1.4. This indicates a degradation of the pectin by treatment at temperatures of over 100° to a more or less constant level—a fairly definite degree of depolymerization.

A further point of agreement with the findings for jelly strength is that all the pectins from orange peel have higher viscosities than those from apple. This is in keeping with recent observations by Svedberg & Gralen [1938] who find that in the fruit juices the mol. wts, determined by ultracentrifugal methods, approximate to 40,000–50,000 for orange pectin, and to 25,000–35,000 for apple pectin.

Relative viscosity and composition are shown in Table VI. Only the first five pectins gave jellies.

Table VI

Source	Method of preparation	Temp. of extraction °C.	Urone %	Non-urone %	Methoxyl %	Relative viscosity 0.5 % pectin
Orange peel II	(d)	60	75.52	12.12	12.36	5.20
Orange peel II	(d 40)	60	78.92	10.31	10.77	3.58
Orange peel II	(a)	95	76.64	14.65	8.71	2.38
Apple	(d)	60	67.72	22.22	10.06	2.10
Apple	(a)	95	70.72	19.44	9.84	1.94
Beet	(a)	95	51.84	40.71	7.45	1.64
Orange peel II	(b 40)	110	74.52	14.02	11.46	1.62
Orange peel II	(b)	110	72.12	16.29	11.59	1.42
Apple	(b 20)	110	64.54	25.47	9.99	1.38
Apple	(b 40)	110	62.27	27.77	9.96	1.36
Apple	(b)	110	62.73	27.42	9.85	1.35
Beet	(d)	60	50.36	41.76	7.88	3.16

As in the case of jelly strength it is impossible to draw definite conclusions with regard to the relation of chemical composition and viscosity. The method of preparation and the material used are factors at least equal in importance to composition of the products.

That previous history is an outstanding factor in determining the possibility of jelly formation is again indicated by results for prepared methyl esters of pectolic and pectic acids. Two typical examples gave the following results:

	Urone %	Non-urone %	Methoxyl %	Relative viscosity	Jelly strength
Methyl ester of pectolic acid	85.64	—	13.37	1.27	Nil
Methyl ester of pectic acid	84.60	2.32	13.08	1.17	Nil

The low viscosity of these products corresponds with their lack of ability to form jellies. Nevertheless, if composition were the only factor determining jelly strength and viscosity, these examples might be expected to give high values since each show high urone, low non-urone and high methoxyl contents. It is evident that the laboratory process of esterification involves degradation of pectolic and pectic acids to products of smaller molecular size.

SUMMARY

1. There is little relation between chemical composition and jelly strength or viscosity of pectins. In general, a high urone content connotes satisfactory jelly strength.

2. Methoxyl content is not a criterion of jelly strength: esters of pectolic and pectic acids prepared by methylation failed to give jellies; the methoxyl contents of prepared pectins bore no relation to their power of forming jellies.

3. The method of preparation of pectins is a vital factor with respect to jelly strength and viscosity. Autoclave preparation in absence or presence of sucrose inhibits jelly formation.

4. Jelly strength and viscosity of the same pectin are closely related and show rapid decreases when the pectin is heated. Viscosity shows a decrease when solution is first effected, but becomes constant after five or more hours.

5. The power of pectin to form jellies depends to some extent on composition, but primarily on molecular size as indicated by viscosity. Any pretreatment which tends to disaggregate the polygalacturonide chain tends also to cause loss of jelly strength.

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CLXXVIII. THE OXIDATION OF CATECHOL AND 1:2:4-TRIHYDROXYBENZENE BY POLYPHENOL OXIDASE

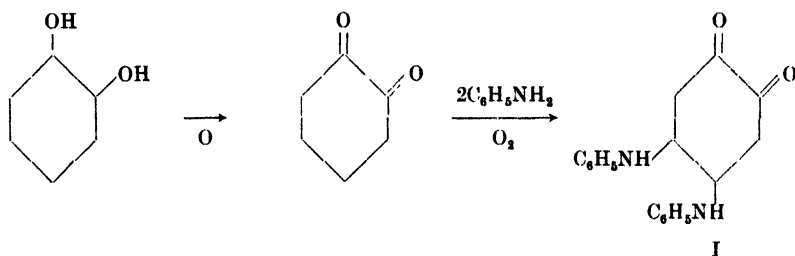
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(Received 21 July 1939)

THE enzyme polyphenol oxidase catalyses the oxidation of *o*-dihydric phenols in general. Oxidation of pyrogallol (1:2:3-trihydroxybenzene), for example, is accompanied by the absorption of 3 atoms of oxygen and the product (purpurogallin) crystallizes out during the reaction [Willstätter & Heiss, 1923]. Catechol, however, does not furnish any directly identifiable product on enzymic oxidation although manometric measurements have shown that 2 atoms of oxygen are absorbed in the reaction [Robinson & McCance, 1925; Pugh & Raper, 1927].

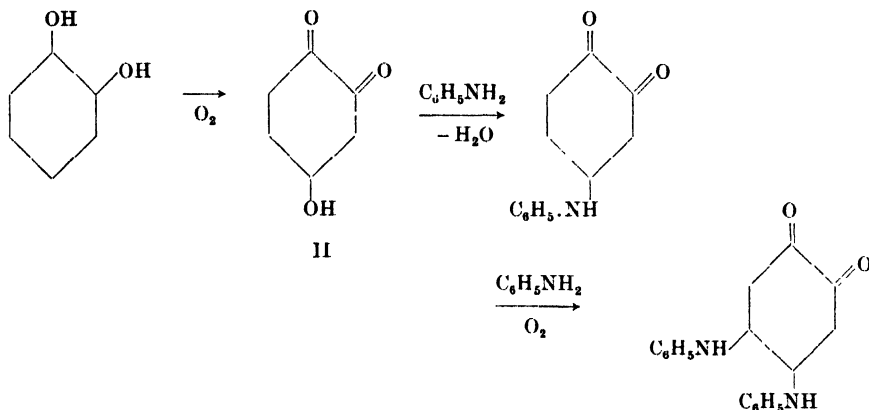
It was suggested by Szent-Györgyi [1925] that *o*-quinone might be the first product of oxidation and Pugh & Raper [1927] obtained experimental evidence in support of this view. They oxidized catechol enzymically in the presence of aniline when dianilo-*o*-quinone (I) separated as a scarlet solid. The following mechanism was therefore proposed:



Subsequently Wagreich & Nelson [1936] found that the requisite 3 atoms of oxygen were absorbed in the reaction. These authors also found experimental evidence suggesting an alternative mechanism for the oxidation process, which possessed the additional advantage of accounting for the second atom of oxygen absorbed in the oxidation of catechol alone. The formation of *o*-quinone postulated by Pugh & Raper only required the absorption of 1 atom of oxygen prior to the spontaneous reaction of the quinone with aniline; the scheme suggested by Wagreich & Nelson necessitated the oxidation of each catechol molecule by 2 atoms of oxygen before reaction with aniline took place.

The experimental evidence for this hypothesis was based on the observation that when catechol was oxidized at pH 6.0 until 2 atoms of oxygen were absorbed, introduction of aniline and fresh enzyme was followed by the gradual absorption of a further atom of oxygen and the formation of dianilo-*o*-quinone. It was

concluded that catechol is probably oxidized to 4-hydroxy-1:2-quinone (II) which then reacts with aniline in the following manner:



In their investigation, Wagreich & Nelson believed that when exactly 2 atoms of oxygen had been absorbed the absence of *o*-quinone could be justifiably assumed. Since, however, oxidation of catechol proceeds to about 2.2 atoms this assumption is by no means certain. Furthermore these authors merely stated that *the same aniloquinone is formed* without giving any information on the important question of how much was produced. The object of the present investigation was to find out more definitely whether *o*-quinone was the enzymic oxidation product of catechol which produced dianiloquinone or whether hydroxyquinone was really the direct product of oxidation reacting with aniline to form dianiloquinone. The experimental procedure adopted consisted of a quantitative study of aniloquinone formation during oxidation of catechol and an examination of the enzymic and autoxidation of 1:2:4-trihydroxybenzene alone and in the presence of aniline.

EXPERIMENTAL

Polyphenol oxidase

The enzyme used by Wagreich & Nelson [1936] was a comparatively crude preparation from mushrooms. The enzyme used in the present work was prepared from the same source, but considerably purified according to the method of Keilin & Mann [1938]. The preparation had a dry weight of 1.8 mg./ml. and Q_{O_2} , 140,000 estimated at 20° .

Catechol oxidations

These were carried out in the usual manner in Barcroft respirometers. The reaction flask received 1 ml. of catechol solution (2.28 mg./ml.), 2 ml. of phosphate buffer (Sørensen, *M*/15, pH 5.9 or 7.1) and a dangling tube containing 0.3 ml. of enzyme concentrate. Addition of aniline to the reacting mixture at any stage of the oxidation was effected by rapidly detaching the reaction flask and fixing a second dangling tube in position containing aniline (30–40 mg.), whilst 0.3 ml. of enzyme solution was added to the reaction fluid. After replacing the apparatus in the thermostat and dislodging the tube, shaking was continued for 4 hr. and a final reading taken next morning. The manipulation could be accomplished without seriously affecting the equilibrium of the system.

Detection and estimation of dianilo-o-quinone

The formation of aniloquinone was easily observed qualitatively by the intense red colour which preceded the separation of the insoluble pigment. Extraction with ether provided a means of detecting traces of this compound when no visible reaction occurred, preliminary experiment having shown that no ether-soluble pigment was produced during oxidation of catechol alone. In quantitative determinations the aniloquinone produced subsequent to aniline addition was estimated colorimetrically after 20 hr. by extraction with ether and adjusting the volume of the extract to 50 ml. The results were expressed as percentages of the quantity of aniloquinone formed when oxidation was carried out entirely in the presence of aniline (Table II).

Preparation of 1:2:4-trihydroxybenzene

The following improved method gave an excellent yield of almost colourless product.

(a) *1:2:4-Triacetoxylbenzene*. *p*-Quinone (15 g.) was mixed with acetic anhydride (45 ml.) and conc. H_2SO_4 (1 ml.) added dropwise (10 min.), the temperature being maintained below 40° by cooling. The pale brown product (20–25 g.) was precipitated by pouring into water. One crystallization from alcohol gave the white crystalline triacetate, M.P. $97\text{--}98^\circ$.

(b) *Hydrolysis of 1:2:4-triacetoxylbenzene*. The acetate (10 g.) was mixed with cold, absolute alcohol (30 ml.) and conc. HCl (1 ml.) added. The mixture was heated for 1.5 hr. in an inert gas stream on a water bath at 80° . The liquid remained colourless or became very pale yellow (if refluxed in air, intense red solutions were obtained). The solvent and acid were removed *in vacuo* leaving a yellow syrup which solidified on treatment with chloroform and scratching. The 1:2:4-trihydroxybenzene (4–5 g.) so obtained, M.P. $135\text{--}136^\circ$, remained quite stable if kept dry.

Oxidations of 1:2:4-trihydroxybenzene

In respirometers, the reaction flask contained phosphate buffer (3.0 ml.) with or without aniline for autooxidations, buffer and enzyme (0.3 ml.) or buffer, enzyme and aniline for enzymic oxidations, the weighed trihydric phenol (4 mg.) being suspended in a dangling tube. For large scale oxidations a quantitative apparatus was constructed having a reaction flask of 1.5 l. capacity connected to a burette system capable of measuring gaseous absorption up to 500 ml. (for general details of this type of apparatus see Jackson, 1938).

Isolation of 5-anilo-4-hydroxy-1:2-quinone

(a) *By enzymic oxidation of 1:2:4-trihydroxybenzene*. The reaction flask received phosphate buffer pH 5.9 ($M/30$, 700 ml.) and aniline (10 ml.), the pH being adjusted to 5.9 by addition of dilute HCl . Enzyme concentrate (50 ml.) was now added and 1:2:4-trihydroxybenzene (1.26 g.) suspended in a weighed tube in the reaction flask, which was then connected to the measuring system and the entire apparatus filled with oxygen. After shaking to temperature equilibrium with the surroundings, the trihydroxybenzene was added to the reaction fluid when oxidation proceeded rapidly, 2 atomic equivalents of oxygen being absorbed in 10 min. Equilibrium was reached after 1 hr. (260 ml. absorbed; required for 2 atoms at 20° , 240 ml.), when the separated solid was filtered off. Yield, 1.5 g. of brownish-maroon powder, M.P. 150° (decomp.), not sharply.

Considerable difficulty was experienced in the purification of this anilo-compound owing to contamination with brown material. The most satisfactory procedure consisted in shaking the crude powder with cold acetone-ether mixture (2:3, 150 ml.) which removed a considerable amount of brown resin. The residue was boiled with acetone (50 ml.) and filtered. A deep violet solid remained which was suspended by a thimble in a reflux apparatus containing acetone so that a hot extraction was carried out. The intense red extract yielded an iridescent, violet solid (500 mg.), M.P. 208° (decomp.), which was dissolved by a similar process in benzene (80 ml.). On cooling, a violet-black microcrystalline solid separated. This was finally purified by repeated crystallization from acetic or propionic acid, from which the hydroxymonoaniloquinone separated in masses of glistening, purple micro-needles, M.P. 210° (decomp.). (Found: C, 67.1; H, 4.5; N, 6.5%. $C_{12}H_9O_3N$ requires C, 67.0; H, 4.2; N, 6.5%.)

The hydroxyaniloquinone was easily removed from ethereal solution by dilute sodium carbonate, in which it formed a yellow solution turning violet on acidification. It dissolved readily in aniline in the cold, followed almost immediately by separation of a light brown solid consisting of yellow micro-needles. On heating the mixture, these dissolved, forming a deep red solution turning brown again on cooling due to separation of the solid, which could be filtered off and washed with light petroleum (200 mg. of hydroxyaniloquinone with 7.5 ml. of aniline gave 100 mg. of product). This substance proved to be an unstable addition compound of aniline and hydroxyaniloquinone since, on heating to 100° or boiling with a little benzene followed by dilution with light petroleum, the aniloquinone was recovered quantitatively. (100 mg. of aniline compound gave 70 mg. of hydroxyaniloquinone; $C_6H_5NH_2 \cdot C_{12}H_9O_3N$ (100 mg.) requires $C_{12}H_9O_3N$ (70 mg.).)

(b) *From autoxidation of 1:2:4-trihydroxybenzene in the presence of aniline.* The procedure was identical with that described under (a) except that no enzyme was used. An oxygen absorption of nearly 4 atomic equivalents took place during 1 hr. (460 ml. found; 480 ml. required) and the solid which had separated from the deep red solution was filtered off. Yield, 2 g. of red powder, decomposing above 200° . Purification was effected by boiling with methyl alcohol (50 ml.) and refluxing the undissolved material with hot acetone as described in (a). The filtrate gave a deep violet solid (500 mg.), M.P. 210° (decomp.), giving no depression in melting point with hydroxyaniloquinone from (a) and behaving in identical manner with aniline, alkali and acid. The methyl alcohol filtrate from above gave a further quantity of red solid (300 mg.) on cooling, which deposited as purple needles of the aniloquinone from acetic acid. A similar colour change from red to purple took place on heating.

DISCUSSION OF RESULTS

The theory proposed by Wagreich & Nelson, of the formation of hydroxyquinone rather than *o*-quinone during the enzymic oxidation of catechol, depends upon two experimental observations. These are, that when catechol is oxidized at pH 6.0 until 2 atoms of oxygen are absorbed, subsequent addition of aniline is followed by the formation of dianilo-*o*-quinone with the absorption of a further atom of oxygen.

The results in Table I show that during oxidation of catechol at pH 7.1, addition of aniline after an absorption of 1.71 atoms of oxygen gives only a trace of aniloquinone and none at all after preliminary oxidation of catechol to 1.79 atoms. At pH 5.9, however, after 2 atoms of oxygen have been absorbed,

Table I. *Catechol oxidations at pH 7.1*

Oxygen absorption at aniline addition (atoms)	Time min.	Total oxygen absorption (20 hr.) atoms	Dianiloquinone
—	—	2.1	—
1.71	34	2.37	+ve
1.79	38	2.20	-ve
1.83	40	2.31	-ve

Table II. *Catechol oxidations at pH 5.9*

Aniline added after min.	Oxygen uptake at aniline addition atoms	Total oxygen absorption 20 hr. atoms	% aniloquinone	Oxygen uptake % of 3 atoms
—	—	2.18	—	—
0	0	2.96	100	100
0	0	3.06	100	100
1	—	—	95	—
3	—	—	79	—
8	1.18	2.76	59	91
28	1.52	3.00	53	100
35	1.71	2.83	51	94
56	1.78	2.79	51	93
97	1.98	2.79	30	93
97	1.99	2.76	27	91
198	2.20	2.83	15	94
198	2.20	2.95	15	98

a deep red colour appears with aniline and further oxygen is absorbed to a final value of 2.8 atoms after 20 hr. Yet only a few flecks of aniloquinone can be seen in the brown reaction fluid. When oxidation is first completed (2.2 atoms) before addition of aniline, no colour develops with the amine and only a small quantity of pigment is subsequently extracted by ether. If dianiloquinone is formed from 4-hydroxy-1:2-quinone and aniline, as Wagreich & Nelson suggest, it follows that comparable quantities of the anilo-compound should be formed when aniline is added at the commencement or at the 2 atom stage of the oxidation of catechol; otherwise the total absorption of 3 atoms of oxygen in the latter case can be of no significance. Quantitative estimation of the aniloquinone produced by addition of aniline at various stages of the oxidation (Table II) shows that the maximum quantity is formed when aniline is present from the commencement of the oxidation, the amount decreasing rapidly at first as addition of aniline is delayed and later diminishing much more slowly. After 2 atoms of oxygen are absorbed (97 min. required, compared with 90 min. in Wagreich & Nelson's experiment) only 30 % of aniloquinone is formed; when 200 min. have elapsed before adding aniline the figure is reduced to 15 %. In the preparation of the standard aniloquinone solutions, it is noticeable that one extraction with ether completely removes the red solid, leaving the aqueous phase clear and colourless; as introduction of aniline into the oxidizing catechol is gradually delayed, subsequent extraction with ether leaves the aqueous liquid more and more brown in colour.

These observations suggest that catechol is first oxidized to *o*-quinone which is unstable and in the absence of aniline rapidly oxidizes to brown material. The composite graph (Fig. 1) shows the oxygen absorption occurring after additions of aniline to oxidizing catechol in experiments in which the aniloquinone was later estimated. The diminution in the rapid uptake following the additions corresponds to the observed fall in the amount of aniloquinone produced.

Furthermore, in all these experiments the total oxygen absorption after 20 hr. is about 3 atoms (Table II) irrespective of the amount of aniloquinone formed. It follows, therefore, that the uptake of an additional atom of oxygen by oxidizing catechol when aniline is introduced at the 2 atom stage, cannot safely be used in formulating the mechanism of aniloquinone formation.

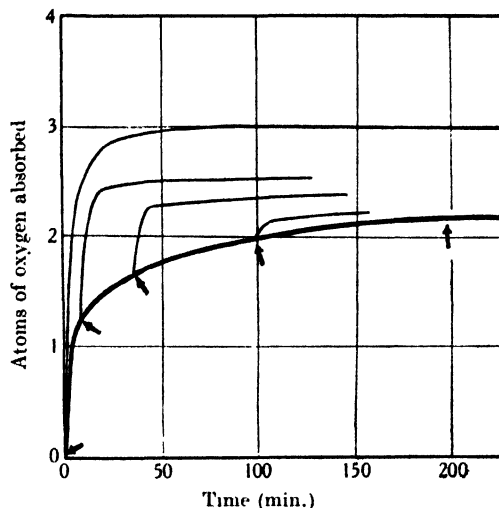
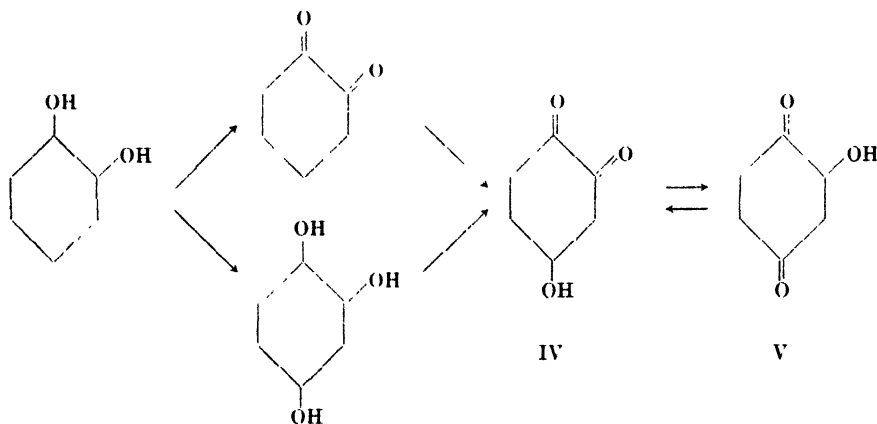


Fig. 1. Composite curve showing the oxygen absorption following introduction of aniline at various stages during the enzymic oxidation of catechol. The points indicated show the time of addition of aniline in different experiments.

The oxidation of catechol to 4-hydroxy-1:2-quinone could hardly take place directly as Wagreich & Nelson suggest. Either *o*-quinone or 1:2:4-trihydroxybenzene would be expected as an intermediate product.



Oxidation of 1:2:4-trihydroxybenzene could proceed either to 4-hydroxy-1:2-quinone (IV) or 2-hydroxy-1:4-quinone (V), which however are tautomeric forms. Although the trihydric phenol autoxidizes quite readily in aqueous solution at *pH* 5-9, polyphenol oxidase exerts a strong catalytic action on the

reaction (curves *a* and *b*, Fig. 2); the enzymic oxidation probably proceeds via the 1:2-quinone. One atom of oxygen is rapidly absorbed with the development of an intense red colour which rapidly turns brown in a few minutes with further slight absorption of oxygen indicating the unstable nature of the quinone. In the presence of aniline and enzyme, 2 atoms of oxygen are rapidly absorbed (curve *c*) but no trace of dianilo-*o*-quinone is formed, the reaction fluid being

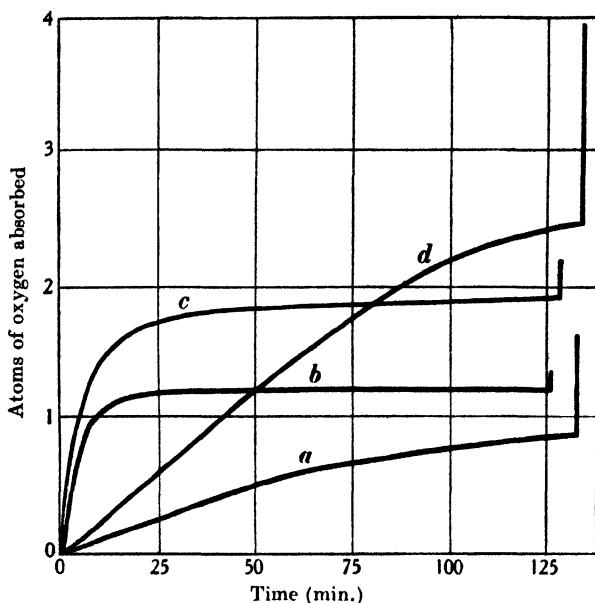
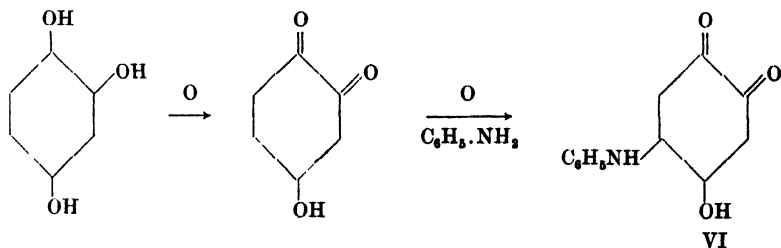


Fig. 2. The enzymic and autoxidation of 1:2:4-trihydroxybenzene alone (curves *b* and *a* respectively), and in the presence of aniline (*c* and *d* respectively). The vertical part of each curve represents O_2 uptake up to 20 hr.

opalescent violet-brown in appearance. The colour is completely removed by ether forming a violet-red solution. The pigment, isolated from large-scale oxidations, crystallizes in purple micro-needles giving analytical figures agreeing with the expected hydroxymonoaniloquinone (VI). Autoxidation of the trihydric phenol in the presence of aniline also gives no dianilo-*o*-quinone and 3.9 atoms of oxygen are absorbed (curve *d*). The reaction fluid is similar in appearance and behaviour to that from the enzymic oxidation and the only product which can be isolated is a considerable proportion of the compound (VI). In view of the oxygen uptake this is quite unexpected and is as yet inexplicable.

The enzymic oxidation of 1:2:4-trihydroxybenzene can therefore be represented in the following manner:



Thus hydroxyquinone cannot accumulate during the enzymic oxidation of catechol owing to its instability, or take any part in the formation of dianilo-*o*-quinone since it reacts with aniline to form a stable hydroxyaniloquinone. There is no evidence, therefore, of an alternative mechanism for the enzymic oxidation of catechol—*o*-quinone must be the initial product. Subsequent oxidation probably consists in the rapid decomposition of the unstable quinone.

SUMMARY

1. The oxidation of catechol by polyphenol oxidase in the presence of aniline has been investigated.

2. The amount of dianilo-*o*-quinone formed diminishes as introduction of aniline into the oxidizing catechol is delayed; the total oxygen absorbed, however, remains approximately constant at 3 atoms per molecule of catechol.

3. Oxidation of 1:2:4-trihydroxybenzene at pH 5.9 is catalysed by polyphenol oxidase. In the presence of aniline, 2 atoms of oxygen per molecule of trihydric phenol are absorbed; autoxidation under the same conditions requires approximately 4 atoms of oxygen. The only product which can be isolated in both cases is a hydroxymonoaniloquinone, no dianilo-*o*-quinone being formed.

4. These results do not support the suggestion that the enzymic oxidation of catechol proceeds to hydroxyquinone, or that the formation of dianilo-*o*-quinone takes place by interaction of aniline with hydroxyquinone or any higher oxidation product of catechol than *o*-quinone.

The author wishes to thank Prof. H. S. Raper for his interest and advice during the progress of the investigation.

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CLXXIX. FACTORS PREVENTING OXIDATION OF ASCORBIC ACID IN BLOOD SERUM

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THE extreme readiness with which ascorbic acid undergoes oxidation *in vitro*, in presence of traces of Cu, leads one to suppose that under the conditions encountered in animal organisms, i.e. in presence of O₂ and of salts of Cu and Fe, this process would also proceed rapidly were it not for the existence of certain protective mechanisms. These mechanisms, which act *in vitro*, but which may also apply *in vivo*, may be classified into two types:

(a) Inhibition of conversion of ascorbic to dehydroascorbic acid by proteins, amino-acids and salts [Kellie & Zilva, 1935; Stotz *et al.* 1937; Barron *et al.* 1937].

(b) Prevention of conversion of dehydroascorbic acid into further irreversibly oxidized products by glutathione and possibly other thiol derivatives [De Caro & Giani, 1934; Borsook *et al.* 1937].

The action of factors of the first class depends on binding by proteins and amino-acids of Cu, which catalyses this process, whilst the action of factors of the second class depends on oxidation-reduction processes causing reconversion of dehydroascorbic acid. No satisfactory explanation of the action of salts has been put forward.

The present paper describes experiments on the action of proteins, amino-acids and salts on oxidation of ascorbic acid *in vitro*.

EXPERIMENTAL

Methods. The systems were prepared in Erlenmeyer flasks, and contained 16 ml. of water or phosphate buffers, 2 ml. of a 0.05–0.1 % solution of ascorbic acid, and 2 ml. of the added substances whose influence was under examination. The concentration given in the tables are those attained in the solution after addition of the substances, e.g. salt. The systems were left in open flasks at a temperature of 25°. At the beginning of the experiment and after a given time (stated in the tables), 1 ml. samples were taken and, after acidification with acetic acid, the ascorbic acid content was determined by titration with dichlorophenolindophenol solution. The figures given in the tables are means of 3–5 determinations. The solutions of ascorbic acid were prepared immediately before the experiment.

In the experiments for the examination of the influence of protein we added horse serum globulin obtained by 1/2 saturation with ammonium sulphate, followed by dissolving of the precipitate and dialysis for 4 days against distilled water. Globulins prepared by other methods and other proteins were also used, but the effect obtained did not differ greatly. Deproteinization was effected with the help of a solution containing 8 % trichloroacetic acid and 2 % metaphosphoric acid.

In the experiments on the influence of blood components, efforts were made to maintain the same relation of the various constituents as in the blood, i.e. the amount of haemolysed corpuscles in 5 ml. of solution corresponds to 10 ml. of whole blood.

The ascorbic acid was generously given to us by Roche, Ltd., Warsaw.

RESULTS

(1) *Effect of proteins.* Our first experiments had as their object the establishment of the least concentration of protein still affecting oxidation of ascorbic acid, as well as to demonstrate whether the inhibitory action of protein was proportional to its concentration. The velocity of oxidation of ascorbic acid in these experiments depended on the concentration of protein as well as on the Cu content of the distilled water used. This was constant in our experiments, and amounted to about 0.01 p.p.m.

Table I. *Influence of serum globulin on oxidation of ascorbic acid*

Conc. of globulin %	Conc. of CuSO ₄ %	mg. ascorbic acid remaining after				
		0	45 min.	120 min.	165 min.	24 hr.
0.002	—	0.625	—	0.33	—	—
0.011	—	0.625	—	0.53	—	—
0.023	—	0.625	—	0.55	—	—
0.063	—	0.625	0.55	—	0.51	0.24
0.125	—	0.625	0.51	—	0.51	0.22
0.625	—	0.625	0.52	—	0.52	0.14
1.250	—	0.625	0.55	—	0.47	0
0.625	0.000125	0.625	0.32	—	0.14	0
0.625	0.000250	0.625	0.33	—	0.13	0
0.625	0.001250	0.625	0.22	—	0.09	0
Control	—	0.625	0.34	0.25	—	0

It appears from Table I that the inhibitory action of serum globulin is evident at a concentration of 0.011 %. With increasing concentration of protein this effect augments only to a certain limit, being smaller with 0.1 % protein than at lower concentrations. In systems to which CuSO₄ was added the concentrations of protein did not suffice to bind the entire amount of Cu, and in these experiments the inhibitory action of protein was not evident.

The effects obtained with whole serum were similar to those with globulin; highly diluted sera had a greater action than undiluted or slightly diluted sera.

In view of the possibility that the preparations of serum globulin taken contained Cu, which might catalyse the reaction of oxidation of ascorbic acid, the ash remaining after combustion of 2 g. protein was dissolved in a dilute solution of H₂SO₄ in triply distilled water, and the solution was added to aqueous solutions of ascorbic acid. Considerable activation of oxidation was found in such systems, for no ascorbic acid could be found after 50 min., as compared with over 12 hr. in the control systems. It may be concluded that the fall in protective action of protein with its increasing concentration is ascribable to its inorganic content, and hence probably to Cu, the presence of which in serum proteins has repeatedly been demonstrated [cf. Guillemot, 1932; Warburg & Krebs, 1927; Mann & Keilin, 1938]. This finding is of considerable interest with reference to the relation of proteins to Cu in the catalysed oxidation of ascorbic acid. Thus we know that proteins bind Cu, which catalyses this reaction. On the other hand, this binding does not totally abolish the catalytic activity of Cu. As was pointed out by Stotz *et al.* [1937], the Cu-protein complex is of such a

type that transformation of Cu^{I} to Cu^{II} , or the reverse, is possible. Cu combined with protein retains its catalytic properties, albeit to a smaller extent than in the ionic state. Stotz *et al.* even consider Cu-protein complexes as a kind of enzyme model.

A comparison of different proteins showed that globulins prepared from the serum of animals immunized to diphtheria did not differ in their activity from those from normal animals. Gelatin, as has already been shown by Stotz *et al.*, has a feeble protective action than has casein, which also acts slightly more strongly than does serum globulin. The experiments with casein had to be performed under somewhat different conditions in view of its insolubility at ordinary pH. Its inhibitory action at the higher pH was, however, greater than that of other proteins studied.

(2) *Effect of amino-acids.* Glycine inhibits oxidation in concentrations of 0.25%. The effect does not diminish with rising glycine concentration, as was the case with proteins. Leucine and aspartic acid were also investigated. These amino-acids are with difficulty soluble in water, but the concentrations taken sufficed to establish a definite inhibitory action on the reaction. Acetamide had no action.

Table II. *Effects of glycine, leucine, aspartic acid and acetamide on the oxidation of ascorbic acid*

	pH	mg. ascorbic acid found after			
		0	1 hr.	2 hr.	24 hr.
Control	7.0	0.44	0.14	0	0
Glycine 0.66 %	7.0	0.44	0.42	0.40	0.18
„ 3.30 %	6.9	0.44	0.43	0.42	0.18
„ 6.60 %	6.5	0.44	0.44	0.43	0.35
Control	7.1	0.44	0.12	—	0
Leucine 0.1 %	7.2	0.34	0.28	—	0
„ 0.5 %	7.1	0.34	0.28	—	0.13
„ 0.9 %	7.0	0.34	0.32	—	0.16
Aspartic acid 0.1 %	7.0	0.34	0.23	—	0.05
„ 0.5 %	7.5	0.34	0.31	—	0.20
„ 0.9 %	7.1	0.34	0.32	—	0.27
Acetamide 0.2 %	7.1	0.34	0.15	—	0
„ 1.0 %	7.1	0.34	0.13	—	0
„ 1.8 %	7.1	0.34	0.19	—	0

(3) *Effect of blood constituents.* As has been mentioned, oxidation of ascorbic acid proceeds much more slowly in serum than in aqueous solutions of the same concentration. The inhibitory effect of undiluted serum is less than that of diluted serum, e.g. 1 : 15.

Oxidation in solutions of haemolysed erythrocytes is also slower than in aqueous solutions. Addition of even small amounts of haemolysed blood to serum causes, however, fairly considerable acceleration of oxidation.

(4) *Effect of salts.* It has been shown by Kellie & Zilva [1935] that oxidation of ascorbic acid is retarded in presence of NaCl. The effect of adding a number of salts, at various concentrations and pH, is given in Table IV.

It appears that of the salts studied only chlorides exert a retarding influence on the reaction. It is of interest that NaF, which inhibits a number of enzymic processes taking place in the organism, has no action in this case. The action of NaCl appears to be related to the catalytic action of copper, as is shown by the results of experiments in which the relative concentrations of Cu and NaCl

Table III. *Effect of blood constituents on oxidation of ascorbic acid*

	mg. ascorbic acid found after					
	0	1 hr.	3 hr.	6 hr.	24 hr.	48 hr.
A. Whole blood	1.0	—	—	—	0.88	0.81
Serum	1.0	—	—	—	0.91	0.74
Laked erythrocytes	1.0	—	—	—	0.35	0.11
Laked erythrocytes + serum	1.0	—	—	—	0.53	0.37
Phosphate buffer pH 7	1.0	—	—	—	0	0
B. Serum diluted 1 : 15	0.5	0.47	0.47	—	0.33	—
Serum diluted 1 : 7	0.5	0.48	0.44	—	0.39	—
Serum diluted 1 : 3	0.5	0.47	0.47	—	0.39	—
Serum undiluted	0.5	0.43	0.42	—	0.16	—
Phosphate buffer pH 7	0.5	0.13	0	—	0	—
C. Serum + water	0.4	0.33	—	0.29	—	—
Serum + laked erythrocytes	0.4	0.25	—	0.05	—	—
Phosphate buffer pH 7	0.4	0.15	—	0.05	—	—

The experiments of group A were performed in test tubes of equal size completely filled with the system, covered with paper and sealed with paraffin wax. Oxidation thus took place under conditions of limited O_2 supply, i.e. that dissolved in the solution. Each system contained 10 ml. of whole blood, serum or laked erythrocytes. In the case of a mixture of serum with erythrocytes 5 ml. of laked erythrocytes + 5 ml. of serum were taken. Three parallel determinations were made in each series. To each test tube 1 mg. of crystalline ascorbic acid was added. The experiments of groups B and C were performed as usual in Erlenmeyer flasks, and contained 16 ml. of serum, 2 ml. of erythrocytes and 2 ml. of ascorbic acid solution.

Table IV. *Influence of various salts on oxidation of ascorbic acid at pH 6.9*

	mg. ascorbic acid found after			
	0	1 hr.	5 hr.	24 hr.
Phosphate buffer control	0.4	0.30	0	0
0.5 N KCl	0.4	—	0.4	0.37
0.5 N NaCl	0.4	—	0.4	0.35
0.5 N $CaCl_2$	0.4	—	0.4	0.31
0.5 N $MgCl_2$	0.4	—	0.4	0.35
0.5 N $(CH_3COO)_2Mg$	0.4	—	0	0
0.5 N $MgSO_4$	0.4	—	0	0
0.5 N Li_2SO_4	0.4	—	0	0
0.5 N Na_2SO_4	0.4	—	0	0
0.5 N KNO_3	0.4	—	0	0
0.5 N NaF	0.4	—	0	0
0.003 % $CuSO_4$ in 1.3 % NaCl	0.4	0.21	—	0
0.003 % $CuSO_4$ in 12.0 % NaCl	0.4	0.40	—	0.40

were varied. The effect of chlorides cannot be ascribed to impurities present in the salts as the action of ordinary preparations of KCl did not differ from that found for samples of KCl specially purified by repeated recrystallizations from water and alcohol.

DISCUSSION AND SUMMARY

The protective action of proteins in the reaction of oxidation of ascorbic acid by atmospheric O_2 is less than might be expected were it not that protein-Cu complexes retain a certain catalytic activity. Natural protein-Cu complexes, such as are obtained from serum, thus act on the one hand as feeble catalysts; on the other hand, they are still able to bind a certain amount of copper or iron, and can in this way protect ascorbic acid from oxidation.

Proteins are not the only constituents of serum having an inhibitory action on oxidation of ascorbic acid; amino-acids and chlorides have a similar action.

The chloride ion is perhaps the more important, for its action is exerted irrespective of the cation, and so may act both in the serum and in the tissues. The action of amino-acids is probably far smaller, in view of their low concentration in serum; it may, however, become considerable during resorption from the digestive tract, and during transport to the tissues.

The authors wish to express their gratitude to Prof. M. Michałowicz for his helpful interest.

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CLXXX. METABOLITES OF CONTRACTING MUSCLE. UTILIZATION OF FUMARATE

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(Received 20 July 1939)

IN opening his Harvey Lecture, Lundsgaard [1938] remarked: "It is an assumption frequently encountered in physiological literature, that the metabolism of the aerobically working muscle is a pure carbohydrate metabolism. That assumption cannot have originated from the experience gained in metabolism determinations during muscular exercise. It is a familiar fact that even heavy muscular exercise can be performed on a pure fat diet, and with a respiratory quotient which indicates a combustion almost entirely of fat... Of course, the fact that hard muscular work can be performed under conditions in which the R.Q. is low, does not disprove the belief that working muscles oxidize carbohydrate only; for the possibility certainly exists that in the organism—presumably in the liver—there is a process of carbohydrate formation from fat, so that the organism as a whole is metabolizing fat alone. However, this purely hypothetical possibility can scarcely explain why the conception of a pure carbohydrate metabolism in the aerobically working muscles is so hard to get rid of, as actually seems to be the case. From this remark perhaps you will anticipate that I think that this conception ought to be discarded."

Lundsgaard's statement applies with particular force to mammalian tissues. Hitherto amphibian muscle has been the stronghold of those who support predominant, if not exclusive, carbohydrate oxidation, and therefore the finding of R.Q. lower than unity in the exercising aerobic frog muscle is all the more remarkable. Gemmill [1934] observed that the R.Q. of the contracting sartorius was 0.90 by a manometric and 0.94 by a volumetric method. Later [1936], using improved instruments, the range found was 0.86–0.98, with an average of 0.90. The same author [1935], working also with aerobic frog muscle, found that the average disappearance of sugar accounted for only 42% of the total energy exchange as calculated from the oxygen consumption. It is clear that these facts cannot be squared with exclusive carbohydrate combustion. The problem then arises, what is the nature of the metabolites concerned?

This question cannot be answered at once. Nothing is known about fat metabolism in the exercising frog, and the first experiments must of necessity be conducted by trial and error. The simplest approach is to find out what substances are removed from solution by a working muscle, and it is important that the tissue chosen for experiment should exercise under completely aerobic conditions, as in the living animal when the steady state has been reached. Hill [1928] has discussed the factors which influence the penetration of oxygen into amphibian muscle, and the criteria laid down by him have been carefully observed.

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EXPERIMENTAL

(1) *Physiological details*

Sartorii from English frogs have been used throughout. A cylindrical glass vessel, holding not more than 5 ml., contains bicarbonate-Ringer, and has sealed through the bottom a platinum hook, to which the distal end of the sartorius is fixed by a small S-shaped piece of platinum wire passing through the muscle. The thicker (pelvic) end of the tissue is fastened with a nickel clip, and is hooked by means of a copper stirrup and wire to an isometric lever. Stimulation takes place via the platinum hook and the isometric lever. The control muscle (from the opposite limb) goes into a similar vessel containing an identical volume (4 ml. usually) of Ringer. Through each vessel is passed a stream of oxygen which has traversed a wash-bottle fitted with a Jena glass filter and filled with Ringer; hence the gas is saturated with water vapour at the temperature of the laboratory, and evaporation cannot occur. In addition, the rate of oxygen flow is regulated by screw clips which ensure that the volume passing through each vessel is the same. The Ringer was composed as follows:

NaCl, 0.6 %; NaHCO_3 , 0.1 %; KCl, 0.01 %; CaCl_2 , 0.01 %; the phosphate buffer was 0.028 % and had pH 7.6; the gas mixture contained 97.5 % O_2 and 2.5 % CO_2 . A standard concentration of $M/200$ was employed for the metabolites.

After dissection the muscles are placed in a watch-glass containing a few ml. of the $M/200$ substrate-Ringer, and left for an hour; then the solution is drained off and replaced, and the muscles soaked for another hour. The muscle to be stimulated is now drained on filter paper and put into the appropriate vessel; after passing the gas mixture for a few minutes, stimulation is begun. The control is treated in a similar fashion. Experiments last for 3–5 hr., during which time the muscle is caused to twitch every 5 sec.: under these conditions, with the tissue exposed to oxygen on both sides, it is in a fully aerobic condition [Hill, 1928]. At the end of the allotted period the stimulated muscle is removed from the solution, drained on bibulous paper, dried at 110° and weighed. Meanwhile, 2 ml. of the liquid are taken from each vessel, and the amount of substrate determined. In this way we arrive at the mg. of fatty acid consumed, over and above the control, per g. dry weight per hour, when the sartorius is stimulated in oxygen at twelve shocks per minute.

(2) *Sources of material*

The bulk of the material was of commercial origin, and carefully purified by several recrystallizations from appropriate solvents. Sorbic acid was synthesized by the method of Doebner [1900]; maleic acid purified by conversion into the anhydride [Kempf, 1906]; acetoacetic acid prepared as sodium salt according to Ljunggren [1924]. The glyceride, α -monocrotonin, appears not to have been made before. Epichlorohydrin on hydrolysis furnishes pure α -monochlorohydrin, which is heated with sodium crotonate in a sealed tube. The resulting oil, which could not be crystallized, boiled at $120^\circ/0.25$ mm. (Found (Weiler): C, 52.8; H, 7.12 %; mol. wt. by hydrogenation, 154.5. $\text{C}_7\text{H}_{12}\text{O}_4$ requires C, 52.5; H, 7.5 %; mol. wt. 160.)

(3) *Methods of estimation*

Unsaturated compounds. These were hydrogenated in Barcroft-Warburg manometers, using colloidal palladium as catalyst. The main vessel contains 2 ml. of the fluid to be analysed, made acid with 1 ml. $N \text{ H}_2\text{SO}_4$. The catalyst

is prepared by dissolving 0.2 g. gum arabic in 100 ml. water, and adding 0.2 g. PdCl_2 [cf. Skita & Franck, 1911]; 0.5 ml. of this solution goes into the side cup of the manometer. Hydrogen is passed through for 15 min., shaking the while, thus driving out most of the air and saturating the catalyst; 15 min. further equilibration are then allowed. The determinations are carried out in a bath at room temperature.

Formic acid. The usual chemical procedures are not nearly sensitive enough when applied to small quantities of this acid. Woods [1936] has, however, shown that *Bact. coli* grown in formate broth develops a powerful enzyme, formic hydrogenlyase, which in the absence of oxygen liberates hydrogen quantitatively from formates. The method is very specific; but as the enzyme is inhibited by CO_2 , bicarbonate was omitted from the Ringer and the amount of phosphate raised to 0.035 %. Dr Woods was kind enough to grow the organism and supply details of his method.

Succinic acid. Estimated with succinoxidase, prepared according to Ogston & Green [1935]. This is the least sensitive of the methods used, and a good deal of practice was required in order to obtain consistent results.

Acetoacetic acid. Steam distillation in the presence of $N \text{ H}_2\text{SO}_4$ breaks down the acid to acetone, which is then trapped in alkaline hypiodite; the excess iodine is titrated, after acidification, with $N/200$ thiosulphate.

Control experiments have been performed to show that no interfering substances diffused out of the muscle, whether stimulated or not. In all cases about 1 mg. of substrate is present in 2 ml. of the fluid to be analysed. The difference in substrate content to be expected between "stimulated" and "control" solutions is about 0.1 mg., or 10 % of the whole. Obviously, then, the experimental error of the analysis must not exceed ± 1 %, for work in which the error is greater than 20 % of the change to be anticipated is not likely to carry much conviction. Muscles larger and thicker than the sartorius cannot be employed, because they become anaerobic when stimulated [Hill, 1928], thus defeating an essential purpose of this investigation.

Table I

No. of exps.	Substrate	mg./g. dry wt./hr.
4	Formic acid $\text{H}.\text{COOH}$	—
—	*Oxalic acid $\text{COOH}.\text{COOH}$	—
2	Acrylic acid $\text{CH}_2.\text{CH}.\text{COOH}$	—
6	Crotonic acid $\text{CH}_3.\text{CH}:\text{CH}.\text{COOH}$	—
2	Tiglic acid $\text{CH}_3.\text{CH}:\text{C}(\text{CH}_3).\text{COOH}$	—
3	Sorbic acid $\text{CH}_3.\text{CH}:\text{CH}:\text{CH}:\text{CH}.\text{COOH}$	—
2	Muconic acid $\text{COOH}.\text{CH}:\text{CH}:\text{CH}:\text{CH}.\text{COOH}$	—
6	Succinic acid $\text{COOH}.\text{CH}_2.\text{CH}_2.\text{COOH}$	—
3	Acetoacetic acid $\text{CH}_3.\text{CO}.\text{CH}_2.\text{COOH}$	—
2	Maleic acid $\text{COOH}.\text{CH}:\text{CH}.\text{COOH}$ (<i>cis</i>)	—
7	Fumaric acid $\text{COOH}.\text{CH}:\text{CH}.\text{COOH}$ (<i>trans</i>)	8.6
2	α -Monocrotonin $\text{CH}_2\text{OH}.\text{CHOH}.\text{CH}_2.\text{O}.\text{CO}.\text{CH}:\text{CH}.\text{CH}_3$	—

* Oxalic acid, even when supplied as a saturated solution of the calcium salt, is toxic to frog muscle.

DISCUSSION

A critical eye, glancing down the list of compounds studied (Table I), might remark the odd choice of material. This is not deliberate; it is simply due to lack of precise methods for estimating the fatty acids and their allies. The requirements are severe: quantities of the order of 1 mg. must be determined with a maximum error of ± 1 %. Very few biochemical methods of any kind reach this

standard. The case of β -hydroxybutyric acid may serve as an illustration. Hubbard [1921], oxidizing small amounts of the acid with dichromate, recovered between 84 and 88 % as acetone. These results are the best that have been obtained; and it is doubtful whether the yield of acetone is really so consistent as Hubbard claims. In my own hands dichromate oxidation has given quantities of acetone varying from 80 to 90 % of the theoretical, and in a recent paper by Shipley & Long [1938] the figures range from 63 to 77 %. In connexion with the present study, oxidation has been carried out using persulphate, permanganate, hydrogen peroxide and a ferrous salt, selenium dioxide, perchlorate, colloidal MnO_2 , bromine and silver oxide: attempts have been made also to dehydrate the acid, after evaporating the solution to dryness, with conc. H_2SO_4 , fuming H_2SO_4 , 100 % orthophosphoric acid and zinc chloride. None of these methods represented an improvement over dichromate oxidation, and this is rather unfortunate, as β -hydroxybutyric acid is quite likely to be a fuel of exercise. A satisfactory procedure for β -hydroxybutyric acid has yet to be found, and the same may be said of all the saturated fatty acids.

That the negative results are truly negative, and not spuriously so, appears to be indicated by the following considerations. First, the sartorius is permeable to some, at least, of the substrates employed. By soaking a pair of muscles in $M/200$ solutions of acrylate, crotonate, sorbate and succinate, it has been found that after 3 hr. these substances pass into the tissue in such proportions as to imply that simple osmotic equilibrium has been attained. In the case of succinate, indeed, rather more goes in than can be accounted for by the 80 % of water which the muscles contain—perhaps in order to satisfy the needs of respiration. Secondly, the unsaturated acids are not merely oxidized to the corresponding keto-acids (which would be reduced by hydrogen and palladium): crotonic acid, for example, does not yield acetoacetic acid, since neither Rothera's test nor distillation into hypiodite yield the faintest trace of acetone. Thirdly, it is unlikely that the acids are oxidized at the terminal methyl group, giving carboxyl; if this were the case, crotonate would change into fumarate, which would disappear in the usual way, and acrylate would also be destroyed. Fourthly, the failure to oxidize α -monocrotonin indicates that preliminary esterification with glycerol is not an essential step in the combustion.

The negative results are not due to seasonal influence, for experiments have been carried out on "summer" and "winter" frogs with consistent lack of success. Nor can they be due to competition by the glycogen-lactic acid system: intraperitoneal injections of 30–40 units of insulin were given to some of the frogs 24 hr. before killing, and although the glycogen (extracted by the method of Kerly [1930], and estimated as sugar "after hydrolysis" with the reagent proposed by Somogyi [1937]) was depleted by this procedure, the sartorii from another batch of frogs treated in the same way were unable to use any of the substrates (except fumarate, of course; but even here no gain in fumarate disappearance was observed with the insulinized muscles). The concentration employed likewise cannot be held responsible, for no disappearance was noted when $M/100$ solutions of crotonate, succinate and acetoacetate were supplied: in any case the utilization of $M/200$ fumarate suggests that this concentration is adequate. The failure to remove these fatty acids means, therefore, that frog muscle is unable to initiate their combustion; and this finding must be regarded as significant.

Nor does the positive result with fumarate appear to be false. The acid is not functioning as a hydrogen acceptor; were this so, the succinate formed would diffuse out of the muscle (since it is not burnt, and the tissue is permeable to it),

but tests of the solution with succinoxidase have proved that no succinate is present at the end of the period of exercise. Moreover, the amount of fumarate metabolized is on a smaller scale than can be conveniently explained on the hypothesis of partial combustion, for calculation shows that the disappearance is about what would be expected on the basis of complete oxidation. Hill [1928] has found that a single maximal twitch of a frog muscle liberates 7×10^{-3} cal. per ml. (i.e. per g. wet wt. roughly). With 720 twitches an hour the heat liberated would be 5.04 cal. per g. wet wt., or, multiplying by 5 (since the muscle contains 80 % water), 25.2 cal. per g. dry wt. Now when fumaric acid is burnt [Kharasch, 1928] it yields 320 kg. cal. per mol., or 2.76 cal. per mg. Hence the amount of heat which might have come from the acid in these experiments is $8.6 \times 2.76 = 23.6$ cal., or 94 % of the total. Too much reliance must not be placed on these figures, and work is now in progress to determine the relation between fumarate disappearance and energy output.

It would be premature to base any wide conclusions upon the limited findings of the work here described. The disappearance of fumarate indicates that frog muscle can use other sources of energy than carbohydrate, but the mystery surrounding the low R.Q. of the exercising sartorius has not been dispelled. Until precise methods are available, we shall remain ignorant of the part played by fats in the economy of muscular exercise.

SUMMARY

1. A number of fatty acids and a glyceride have been supplied to frog sartorii stimulated twelve times a minute under completely aerobic conditions.

2. Formic, oxalic, acrylic, crotonic, tiglic, sorbic, muconic, succinic, maleic and acetoacetic acids, together with α -monocrotonin, were not used in significant amounts during exercise.

3. Fumaric acid disappears at the rate of 8.6 mg. per g. dry wt. per hr.: a rough calculation indicates that the amount of fumarate consumed, if completely oxidized, would account for 94 % of the total heat developed by the muscle. The calculation is not considered to be very reliable, and further experiments are in progress.

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CLXXXI. STUDIES ON DIFFUSING FACTORS. III

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(Received 24 July 1939)

THE presence of diffusing factors in snake venoms has been demonstrated by Duran-Reynals [1936, 1939]. The immediate effect of intracutaneous injection of indicator solutions containing rattlesnake venom is similar to that of solutions containing testicular diffusing factor [Madinaveitia, 1939], namely, an almost instantaneous flattening of the resulting bleb with rapid spread of the indicator through the skin tissue. About 1 hr. after injection, however, a difference is observed between the two; the rate at which the area of spread increases becomes extremely slow in the case of testicular diffusing factor while it is still relatively rapid with the rattlesnake venom solution (Fig. 1). This secondary

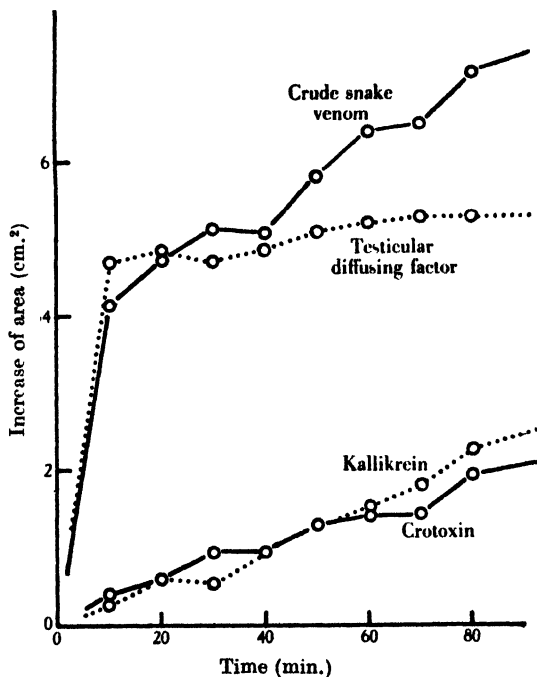


Fig. 1. Comparison of 1:3 dilutions in isotonic haemoglobin solution of solutions of 1 mg./ml. of crude snake venom and crystalline crotoxin, testicular diffusing factor 300 units/ml. and kallikrein ("Padutin" Bayer).

spreading effect has never been observed with testicular diffusing factor and it suggests that the diffusing factor in rattlesnake venom is accompanied by another substance capable of causing a slow spread of indicator through skin tissue. Substances giving rise to a slow spread are known to exist, e.g. kallikrein [Christensen, 1939; Madinaveitia, 1939]. Intracutaneous injection of snake

venom produces local oedema and eventually necrosis, and it is possible that the slow spread above-mentioned might arise as a result of these effects. The occurrence of local reactions is much reduced when, instead of working with normal rabbits, animals immunized against the venom are employed.

Duran-Reynals [1939] has compared the increases in area of spread due to intracutaneous injections of rattlesnake venom in normal and immune rabbits. From his results he deduced that immunization inhibits or suppresses the effect both of the diffusing factor and of the substance responsible for the local reactions. If, however, his experimental results are plotted graphically (Fig. 2)

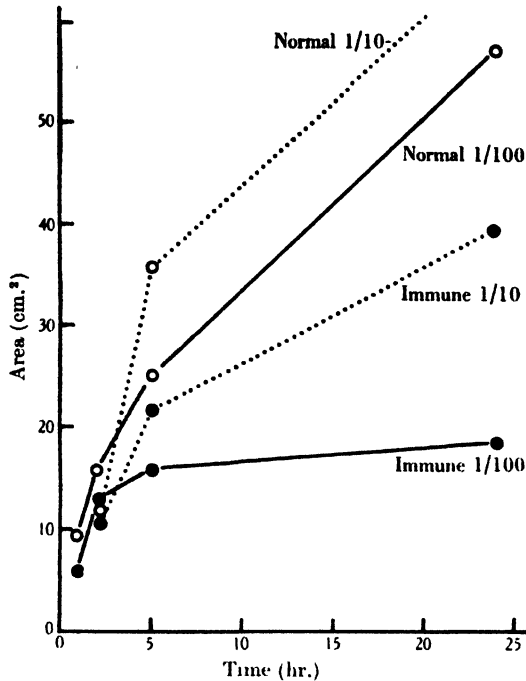


Fig. 2. Time-response curve of rattlesnake venom in normal and immune rabbits. Experimental results of Duran-Reynals [1939]. Indian ink as an indicator.

it is observed that the time-response curves obtained with immune rabbits are like those given by testicular diffusing factor preparations and show a very rapid initial spread followed by a very slow one. Immunization has suppressed not the initial rapid spread, but only the secondary one. Since some local reaction occurs when relatively strong solutions of venom are injected into immune animals, a certain degree of secondary spread is observed in such cases.

Slotta & Fraenkel-Conrat [1938] have isolated in crystalline form crotoxin, the neurotoxic constituent of rattlesnake venom. Through the kindness of Prof. Slotta, to whom my thanks are due, a sample of the crystalline toxin was placed at my disposal for examination. Intracutaneous injection of crotoxin produces a slow spread of the injected fluid but the initial rapid spread characteristic of diffusing factors is entirely absent (Fig. 1). It therefore appears that the neurotoxic constituent and the diffusing factor in rattlesnake venom are distinct from each other. Crotoxin possesses the lecithinase properties of the

crude venom but the blood-clotting properties of the latter are absent [Slotta & Fraenkel-Conrat, 1938]. Whether or not the blood-clotting properties are related to the diffusing factor is not yet known. The blood-clotting power of snake venoms has been attributed by Eagles [1937] to their proteolytic activity.

Some bacteria of the gas gangrene group secrete proteolytic enzymes in culture media. Maschmann [1938] showed that one of these enzymes, collagenase, is specific for gelatin and he suggested that it might be responsible for the changes in tissue following invasion by bacteria of the gas gangrene group. It was therefore of interest to investigate the possibility that diffusing factor activity might be associated with an enzyme of the type of collagenase or the proteolytic enzymes of snake venom. This does not appear to be the case; testicular extracts with a high diffusing activity appear to be without action on gelatin. To conclude from this experiment that diffusing activity is not associated with a proteolytic enzyme involves the assumption that diffusing factors from different sources are identical or at least very closely related in their mode of action. Such an assumption seems reasonable on account of the apparent identity of their effects and the close similarity in their chemical properties as far as they have been investigated.

The methods hitherto used for the purification of diffusing factors from various sources depend on the fact that these factors are not precipitated by neutral lead acetate or by half-saturation with $(\text{NH}_4)_2\text{SO}_4$.

The inaccuracy of the biological test method makes it very difficult to determine the relative activities of different fractions obtained during concentration, since it only indicates with certainty whether any one fraction is ten times more or less active than another. This fact together with the instability of diffusing factors, limits the choice of concentration methods. The possibility of concentration by filtration through a column of aluminium oxide has been previously reported [Madinaveitia, 1939]. The results indicated that the testicular diffusing factor is to some extent adsorbed on the alumina used. It has now been found that *ortho* aluminium hydroxide C (alumina C_γ) quantitatively adsorbs the active constituents both from testicular extracts previously purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and from dialysed *Clostridium Welchii* filtrates. Of the various eluents tried Na_2CO_3 and Na_2HPO_4 were most satisfactory and removed most of the adsorbed material. Removal of most of the material is necessary on account of the above-mentioned inaccuracy of the assay method; the non-eluted material cannot be tested and it is impossible to decide whether or not the eluate contains the main bulk of the active material. By two successive adsorptions and elutions a preparation was obtained from bull testicle which has, as far as could be judged from the biological test, most of the activity of the starting material with only 0.3% of its N content. This preparation appears from its chemical properties to be entirely protein in character.

EXPERIMENTAL

Adsorption of testicular diffusing factor on alumina C_γ

The starting material was a concentrate obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation of crude testicular extract. 75 ml. of this concentrate containing about 1 mg. N/ml. were buffered with 25 ml. of an *M*/3 acetate buffer pH 5.5.

To 20 ml. portions of this buffer solution varying amounts of the alumina gel were added and the volume made up to 25 ml. in each case. After 15 min. at room temperature with frequent shaking the alumina was centrifuged off and

the N contents of each of the supernatants determined. Fig. 3 represents the percentages of N adsorbed by different amounts of adsorbent. Between pH 3 and 6 (acetate buffer) variation of the pH at which adsorption takes place has practically no influence on the amount of material adsorbed by a given quantity of adsorbent.

Besides alumina C_γ other adsorbents have also been tried in the following way: to 10 ml. of the buffer solution 0.5 g. of adsorbent was added and, after 15 min., the solution was centrifuged and the N content of the supernatant estimated. The results thus obtained are given in Table I.

Table I

Adsorbent	% of N adsorbed
Kaolin	45
Merck alumina	13.5
Fuller's earth	51
Bauxite	23
Frankonite	43

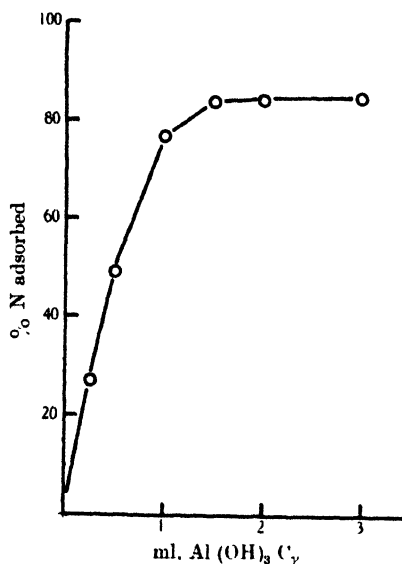


Fig. 3.

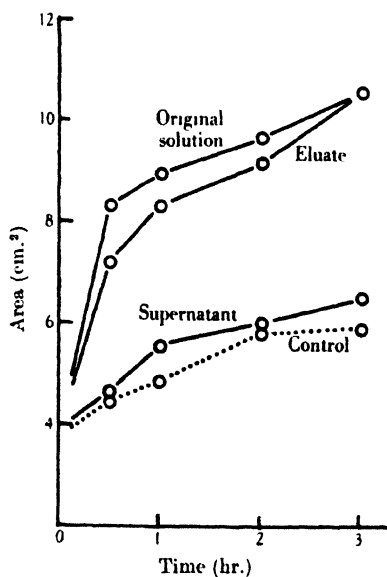


Fig. 4.

Fig. 3. Adsorption of purified testicular diffusing factor on alumina C_γ .

Fig. 4. Adsorption of testicular diffusing factor on alumina C_γ and elution with $M/15 \text{ Na}_2\text{HPO}_4$. Activity of the different fractions.

In order to find the best eluent for the alumina gel adsorbates, portions of 10 ml. of the buffered solution used in the previous experiment were treated with 2 ml. of alumina C_γ . The adsorbate was centrifuged after 15 min. and eluted by shaking for 10 min. with 10 ml. of a $M/15$ solution of the eluent. The results thus obtained are recorded in Table II.

Table II

$M/15$ eluent	CH_3COOH	KH_2PO_4	NaHCO_3	Na_2HPO_4	Na_2CO_3	H_2O
% of adsorbed N eluted	59	17.5	11.5	48	45	1

Although *M*/15 acetic acid appears on this basis to be the best eluent it is unwise to use it since the acidity may partially destroy the activity.

Biological assay. To 75 ml. of the unbuffered solution used in previous experiments (original solution) 25 ml. of alumina gel were added and kept for 2 hr. with frequent shaking. The adsorbate was then centrifuged and washed with 20 ml. of water. After centrifuging again the washing water was added to the supernatant of the adsorption (supernatant). The adsorbate was eluted 4 times with 20 ml. of *M*/15 Na_2HPO_4 and the mixed eluates made up to 100 ml. (eluate). The relative amounts of N in each fraction are shown in Table III.

Table III

Fraction	N content
Original solution	100
Supernatant	18.5
Eluate	69
Non-eluted (by difference)	12.5

For testing purposes each of these fractions was diluted 1 : 10 with an isotonic solution of haemoglobin. Fig. 4 represents the spread of haemoglobin due to each fraction. The technique used for injection and measurement of area was that already described [Madinaveitia, 1938]. Three rabbits were used and each of them was given two injections of each of the solutions under test. The results plotted are the average of the six determinations.

*Preparation of concentrates of testicular diffusing factor by
adsorption methods*

An aqueous extract of dry testicle powder (100 g.) was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation [Madinaveitia, 1939]. The resulting solution (3 l. containing 140 mg. N) was adsorbed on 100 ml. alumina C_γ . After stirring for 1 hr. the adsorbate was allowed to sediment and the supernatant reabsorbed with a further 50 ml. alumina gel. The adsorbates were separated from the supernatant (which contained 38 mg. N) by centrifuging, and were washed by shaking with 50 ml. water which removed 38 mg. of the adsorbed N. They were then eluted by shaking with two portions of 100 ml. 1 % Na_2CO_3 . Elution was completed by shaking with two further portions of 50 ml. of the same eluent. The combined eluates which contained 57 mg. N were adjusted to pH 5.5 with 3 *N* acetic acid. This solution was adsorbed on 50 ml. alumina gel, the adsorbate separated and the supernatant reabsorbed with a further 25 ml. alumina. After separating the supernatants (which contained 7 mg. N), the adsorbate was washed with water which removed 0.7 mg. N. The active material was then eluted by shaking three times with portions of 20 ml. *M*/15 Na_2HPO_4 . The eluates were dialysed against running tap water for one day and then against frequent changes of distilled water for two further days (ice box). The volume of the solution was finally made up to 100 ml. It had 26.4 mg. N, about 0.3 % of the N content of the original crude testicular extract. Biological assay indicated that this preparation had the whole activity of the crude extract and that no fraction having any appreciable diffusing power had been rejected.

The final preparation thus obtained gave precipitates with nitric acid, trichloroacetic acid, salicylsulphonic acid, tannic acid, metaphosphoric acid and uranium acetate. The biuret, xanthoproteic and glyoxylic reactions were positive. After alkaline hydrolysis the test for sulphur was positive. The ash content of the dried material was 1 % and it contained 17.2 % N.

Determination of the Hausmann numbers of this protein according to Thimann [1926] gave the following results: amide-N, 10.1 %, humin-N, 2.2 %, basic N, 27.5 %, amino- and non-amino-N, 63.7 %. These figures are within the limits of those obtained for other proteins.

*Concentration of the diffusing factor filtrates of cultures of
Clostridium Welchii*

Cultures of *Cl. Welchii* were made in a meat broth by Dr D. McClean of the Lister Institute (Elstree) to whom the author is greatly indebted. 2 l. of a culture filtrate having 6 ml. N/ml. were reduced to 250 ml. by evaporation *in vacuo* (23°). In order to find whether or not the active constituents would dialyse this concentrated solution was dialysed against 500 ml. of distilled water (cellophane membrane). After 24 hr. the liquid outside the bag had been reduced to 200 ml. and was completely devoid of any diffusing activity. Dialysis was then continued against running tap water for 2 days. The volume inside the bag increased to 1300 ml. After keeping it for some days in the ice box an inactive precipitate separated. This was filtered off and the clear filtrate had 2.25 mg. N/ml., i.e. about 25 % of the N content of the starting material. This preparation gave copious precipitates with tannic acid and uranium acetate. Lead acetate (basic or neutral) trichloroacetic acid and salicylsulphonic acid only produced slight precipitates.

Adsorption of Cl. Welchii diffusing factor on alumina C_γ

To 5 ml. quantities of the dialysed culture filtrate 1, 5 and 10 ml. of alumina gel were added and in each case the volume was made up to 15 ml. with water. After 30 min. the precipitate was centrifuged. The N contents and spreading activities of the supernatants are recorded in Table IV.

Table IV. *Average of the results obtained in double determinations in two rabbits*

ml. of dialysed culture filtrate	ml. Al(OH) ₃	ml. H ₂ O	% N adsorbed	Area of spread cm. ²	Increase of area (cm. ²)
5	0	10	—	5.43	2.34
5	1	9	38	4.83	1.74
5	5	5	54	3.36	0.27
5	10	0	56	3.34	0.25
Control	—	—	—	3.09	—

The whole of the active constituents have been adsorbed by 5 ml. of alumina gel, but it is doubtful whether 1 ml. is enough to adsorb the bulk of active material.

When working with bacterial diffusing factors there is always the danger that the results of the biological assay are complicated by secondary spread due to the toxin or some other constituents of the filtrate. In order to avoid this difficulty a slight variation of the method of assay has been made. The substance under test is suitably diluted with an isotonic solution of haemoglobin. 0.3 ml. of this dilution is intracutaneously injected into the shaven flank of a rabbit. The area of spread is measured once every minute during the first 5 min. following injection and the figures so obtained are averaged. Comparison can be made between the results obtained with dilutions of any two given preparations in a fashion similar to that previously described [Madinaveitia, 1938]. The comparison can be made either between the whole areas or between the increase of area relative to a control injection without diffusing factor.

In order to determine whether the adsorbed material could be eluted by Na_2HPO_4 as in the case of the testicular diffusing factor, a sample of 5 ml. of the dialysed filtrate was adsorbed with 5 ml. of alumina gel. After 15 min. at room temperature the precipitate was removed by centrifuging. The adsorbate was washed with 5 ml. of water, again centrifuged and then eluted by two portions of 5 ml. of $M/15 \text{ Na}_2\text{HPO}_4$. The activities of each of these fractions are shown in Table V where the figures represent the average of double determinations in two rabbits.

Table V

Fraction	Area of spread cm. ²	Increase of area cm. ²
Original solution diluted 1/1	7.22	3.95
Supernatant of the adsorption	3.42	0.15
Water washings	3.46	0.19
Eluates	7.30	4.03
Control	3.27	—

Comparison of the proteolytic and diffusing activities of Cl. Welchii filtrates and of testicular extracts

The substrate used for determining proteolytic activity was a 5% aqueous solution of gelatin. Solutions containing 8 ml. substrate and 2 ml. enzyme were incubated at 38° for 24 hr., toluene being added as a preservative. The increase in carboxyl groups as determined by titration of 2 ml. quantities of these solutions with $M/20 \text{ KOH}$ [Willstätter & Waldschmidt-Leitz, 1921] was taken as a measure of proteolytic activity. Controls without gelatin and of gelatin without enzyme were uniformly negative.

The crude testicular extract used was prepared by grinding up 1 g. dry testicle powder with sand and water in a mortar. The volume was made up to 20 ml. and the insoluble material separated. The supernatant was coagulated by addition of one drop of $M/10$ acetic acid and filtered. The preparation of the other solutions tested is described elsewhere. The diffusing activity was determined in 1/3 dilutions of the different materials. The results obtained are recorded in Table VI.

Table VI

	Proteolytic activity increase of ml. $M/20 \text{ KOH}$	Diffusing activity increase of area of spread (cm. ²)
<i>Cl. Welchii</i> filtrate	2.43	1.89
<i>Cl. Welchii</i> dialysate	1.60	2.13
Crude testicular extract	0.02	2.37
Purified testicular extract	0.01	2.69

SUMMARY

1. Crude rattlesnake venom contains a factor similar to testicular diffusing factor. Accompanying it is another factor which causes a slow spread of indicators through skin. Rabbits immunized against the venom respond only to the diffusing factor.

2. Crystalline crotoxin does not show the characteristic spreading properties of diffusing factors although it causes a slow spread of indicators through skin.

3. Although crude rattlesnake venom and *Cl. Welchii* filtrates have proteolytic activity it is unlikely that this is associated with the diffusing factors since testicular extracts have no such activity.

4. The diffusing factors from testicle and *Cl. Welchii* are strongly adsorbed by alumina C_γ from which they may be conveniently eluted with sodium carbonate or disodium hydrogen phosphate. The similar behaviour of the factors from these sources suggests that they are closely related or even identical proteins.

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CLXXXII. TRANSAMINATION IN PIGEON BREAST MUSCLE

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D. M. NEEDHAM [1930] first observed that in pigeon breast muscle added glutamic acid disappears from the tissue while the concentration of amino groups, as measured by the Van Slyke method, remained unchanged. She concluded that the amino group of glutamic acid is transferred to "some reactive carbohydrate residue" to form a new amino-acid. Braunstein & Kritzmann [1937; 1938] succeeded in elucidating this reaction in detail. They showed that glutamic acid reacts as follows:

(1) Glutamic acid + α -ketonic acid \rightleftharpoons α -ketoglutaric acid + amino-acid. The "reactive carbohydrate residue" of Needham is thus an α -ketonic acid. The reaction is reversible and proceeds with great rapidity under suitable conditions. It is a general reaction, occurring in almost every animal tissue, and has been termed "transamination" [Schaeffer & Le Breton, 1938; Cohen, 1939; Braunstein, 1939]. The widespread occurrence and the rapid rate of transamination suggest that the reaction plays an important part in tissue metabolism, although its significance is as yet by no means clear.

The detailed study of transamination has been hampered by the lack of suitable analytical methods. The procedure used by Braunstein & Kritzmann [1937] does not appear to be very satisfactory for the authors themselves [1938] question their analytical data in several instances. Since the determination of glutamic acid synthesis in the presence of α -ketoglutaric and amino-acids is one of the ways of measuring transamination, a more reliable method for glutamic acid determination was recently worked out by the author [1939].

The preliminary experiments with this method showed that although most tissues are capable of bringing about transamination, the different tissues show great differences in detail. In muscle, for example, only three amino-acids, *l*(+) glutamic, *l*(-) aspartic and *l*(+) alanine react rapidly; two others, *dl*- α -aminobutyric and *l*(+) valine react slightly; while the other amino-acids do not react appreciably. In kidney, on the other hand, many more amino-acids react.

This paper is confined to a study of transamination in pigeon breast muscle. Experiments on the rates of transamination in this tissue and the reactivity of different amino-acids are reported.

Procedure and methods

Principle of the procedure. For the study of transamination it is desirable to separate this reaction from other reactions in which amino- and α -keto-acids take part. Many of the other reactions require molecular O₂ and they can therefore be eliminated by working under anaerobic conditions. Since it is simpler we have preferred this method to the use of inhibitors. Comparative experiments showed that the results obtained under anaerobic conditions are not essentially different from those obtained aerobically in the presence of bromoacetate [Braunstein & Kritzmann, 1937], an inhibitor which prevents certain

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side reactions. Neither method however eliminates the side reactions completely, for glutamic acid slowly disappears anaerobically, as well as aerobically in the presence of iodoacetate, without the addition of ketonic acids. The mechanism of this reaction is not clear; it may be due to transamination to ketonic acids provided by the muscle. The rate of the reaction however is relatively slow so that it generally does not interfere with experiments on transamination of added substrates.

Transamination was studied by the determination of glutamic acid formed on the addition of α -ketoglutaric acid and amino-acids. In all experiments a blank was carried out with α -ketoglutaric acid alone to determine the glutamic acid formation from preformed NH_2 -donators (NH_3 or amino-acids). The values of the "blanks" in most experiments were considerable, showing that a relatively high concentration of NH_2 -donators is present in pigeon breast muscle.

An increased glutamic acid formation from added α -ketoglutaric acid and amino acids may be considered as conclusive proof of transamination. Euler *et al.* [1939] studied the disappearance of oxaloacetic acid in the presence of amino-acids and they concluded that transamination occurs when the added amino-acids increased the rate of disappearance of oxaloacetic acid. This type of experimentation may suggest that transamination takes place, but cannot provide conclusive proof. The demonstration of aspartic acid formation in this reaction would in our view be the only conclusive evidence of transamination.

Experimental procedure. Pigeon breast muscle was chilled immediately after the death of the animal and finely divided in a Latapie mincer. The minced muscle was suspended in 0.10 *M* phosphate buffer, pH 7.4. Unless otherwise stated the muscle suspension used in the different experiments consisted of 1 part muscle plus 7 parts phosphate buffer. The suspension (3 ml.) was pipetted into the main compartment of conical Warburg manometer flasks provided with a side arm, which contained the substrate, and a centre well which contained a stick of yellow phosphorus. The substrates were used as neutral 0.2 *M* solutions (0.4 *M* for *DL*-amino-acids) and usually added in amounts to give a final concentration of 0.017 *M*. Substrates which were soluble with difficulty were weighed directly into the side arm of the cup.

Anaerobic conditions were maintained by filling the vessels with N_2 and by the yellow phosphorus in the centre well. The vessels were shaken at 40° and unless otherwise stated the substrate was added from the side arm after 5 min. shaking. At the end of the experimental period the flasks were detached and 1 ml. 10% H_2SO_4 added to stop the reaction. The solutions were washed into graduated cylinders with 6–7 ml. distilled water, 1 ml. 10% Na_2WO_4 was added and the solutions made up to a volume of 15–20 ml. The solutions were filtered and an aliquot of the clear filtrate employed for the glutamic acid determination.

Analytical procedure. Glutamic acid was determined by the method recently described by the author [1939]. Interfering succinic acid, and malonic acid when added, were removed by ether extraction before the glutamic acid determination. The aliquot of the clear filtrate was acidified with 2 ml. 10% H_2SO_4 and extracted with ethyl ether for 2 hr. The ether was removed from the aqueous phase and the latter brought to a pH of approximately 5 by the addition of alkali in the presence of a drop of bromocresol purple. The glutamic acid in the aqueous phase was then determined.

The results are expressed as $\mu\text{l.}$ glutamic acid (147 mg. glutamic acid being equivalent to 22,400 $\mu\text{l.}$). This procedure was used since the glutamic acid was determined as $\mu\text{l. O}_2$ (1 $\mu\text{l. O}_2$ is equivalent to 2 $\mu\text{l.}$ glutamic acid).

RESULTS

Glutamic acid formation from amino-acids and α -ketoglutaric acid

The question was first investigated as to which amino-acids react with α -ketoglutaric acid to form glutamic acid. In these experiments a relatively long incubation period of 40 min. was chosen to allow for the formation of measurable amounts of glutamic acid from slowly reacting amino-acids. The results of these experiments are listed in Table I.

Table I. *Glutamic acid formation from α -ketoglutaric acid and different amino-acids*

Substrate conc. 0.017 *M* (for *dl*-amino-acids, 0.034 *M*). N_2 . Yellow phosphorus. 40°.

Amino-acid added	Period of incubation min.	μ l. Glutamic acid formed		Increase in glutamic acid due to added amino-acid μ l.	% of added α -keto-glutaric acid converted into glutamic acid
		Without amino-acid	With amino-acid		
<i>l</i> (-)Aspartic acid	40	161	815	654	49
<i>l</i> (+)Alanine	40	96	636	543	40
<i>l</i> (+)Valine	40	96	220	124	9
<i>l</i> (-)Phenylalanine	40	96	170	74	—
<i>l</i> (-)Tyrosine	40	142	204	62	—
<i>l</i> (-)Cysteine	40	94	152	56	—
<i>d</i> (-)Valine	40	96	149	53	—
<i>l</i> (-)Histidine	40	96	146	50	—
<i>d</i> (+)Histidine	40	96	140	44	—
<i>l</i> (-)Leucine	40	142	180	38	—
<i>d</i> (-)Alanine	40	130	160	30	—
<i>l</i> (+)Citrulline	40	142	168	26	—
<i>l</i> (+) <i>iso</i> Leucine	40	142	166	24	—
<i>l</i> (-)Methionine	30	138	156	18	—
<i>l</i> (+)Arginine	40	96	99	3	—
Glycine	40	96	95	—	—
<i>l</i> (+)Ornithine	40	142	104	—	—
<i>l</i> (-)Proline	40	142	142	—	—
<i>d</i> (+)Phenylalanine	40	96	92	—	—
<i>l</i> (-)Tryptophan	40	142	124	—	—
<i>dl</i> -Serine	40	142	140	—	—
<i>dl</i> -Lysine	30	138	113	—	—
Homologous series					
<i>dl</i> -Alanine	40	130	564	434	32
<i>dl</i> - α -Aminobutyric acid	40	130	266	136	10
<i>dl</i> - α -Aminovaleric acid	40	130	145	15	—
<i>dl</i> - α -Aminohexonic acid	40	130	150	20	—

Of the 21 different amino-acids tested, *l*(-)aspartic acid and *l*(+)alanine are the only ones which show a large glutamic acid formation; *dl*- α -aminobutyric acid and *l*(+)valine show a slight activity. None of the remaining amino-acids is significantly active.

These results differ in various points from those reported by Braunstein & Kritzmann [1938] whose experiments were carried out aerobically in the presence of bromoacetate. In order to ascertain whether the differences in the results are due to differences in experimental conditions, transamination was also studied under conditions similar to those of Braunstein & Kritzmann. It is seen from Table II that the results obtained aerobically in the presence of iodoacetate are essentially the same as those obtained anaerobically. If anything these experi-

Table II. *Glutamic acid formation from α -ketoglutaric acid and different amino-acids under aerobic conditions in presence of iodoacetate*

Substrate conc. 0.016 *M*. Iodoacetate conc. (final), 0.002 *M*. (Iodoacetate plus muscle suspension incubated 15 min. before substrate added). 1 part minced muscle plus 5 parts phosphate buffer. Reaction time, 60 min. Air.

Amino-acid added	Glutamic acid formed μ l.	Increase in glutamic acid due to added amino-acid μ l.	% of added α -ketoglutaric converted into glutamic acid
—	92	—	—
<i>l</i> (+)Valine	98	6	—
<i>d</i> (-)Valine	33	—	—
<i>d</i> (+)Histidine	98	6	—
<i>l</i> (-)Histidine	55	—	—
<i>l</i> (+)Arginine	127	35	—
<i>l</i> (+)Alanine	450	358	27
No iodoacetate			
<i>l</i> (+)Alanine	214	122	9

ments show that the formation of glutamic acid is greater under anaerobic conditions. The inhibitory effect of iodoacetate on the aerobic disappearance of glutamic acid formed from *l*(+)alanine and α -ketoglutaric acid is seen from Table II. This is in agreement with the findings of Braunstein & Kritzmman [1937] who first reported this effect with bromoacetate.

Braunstein & Kritzmman [1938] found 14 different amino-acids to react with α -ketoglutaric acid in pigeon breast muscle, but the authors themselves question their results for 3 amino-acids. Of these amino-acids only the findings for alanine are confirmed by our experiments. From our data previously discussed it follows that the discrepancy between our results and those of Braunstein & Kritzmman cannot be attributed to a difference in experimental procedure. The different results may however be explained by the omission of a suitable control by Braunstein & Kritzmman. As can be seen from Table I α -ketoglutaric acid when added to a muscle suspension forms considerable amounts of glutamic acid in the absence of any added amino-acid. The failure of Braunstein & Kritzmman to take this into account is the chief reason for the high values obtained for all the amino-acids. An additional factor is to be found in the method used by Braunstein & Kritzmman for the determination of glutamic acid. The method employed by these workers measures the $\text{NH}_2\text{-N}$ which is insoluble in alcohol after the addition of $\text{Ba}(\text{OH})_2$ and thus would include aspartic acid and possibly other amino-acids. The high values for certain of the amino-acids, e.g. cystine, reported by these workers is probably due to the insolubility of these amino-acids under the conditions used for the analysis.

On the other hand, of all the amino-acids studied we find *l*(-)aspartic to be the most active. Braunstein & Kritzmman have reported no data for this amino-acid, presumably because their method does not permit the differentiation between glutamic and aspartic acids.

None of the amino-acids of the *d*-series tested was found to be active (Table I).

Effect of ketonic acids on the anaerobic disappearance of glutamic acid

Under anaerobic conditions glutamic acid slowly disappears when added to pigeon breast muscle [Needham, 1930; Braunstein & Kritzmman, 1937; Cohen, 1939]. Braunstein & Kritzmman [1937] showed that the disappearance of glutamic acid is markedly accelerated if pyruvic acid is added, and that the disappearance of glutamic acid is accompanied by the formation of equivalent

amounts of alanine. These authors thus demonstrated the reversibility of transamination.

In order to determine whether pyruvic acid can be replaced by other ketonic acids, the effects of a series of α - and other keto-acids on the anaerobic disappearance of glutamic acid were studied. If the activities of the members of the homologous series of α -ketomonocarboxylic acids are compared (Table III)

Table III. *Disappearance of added glutamic acid in the presence of ketonic acids*

Substrate conc. 0.017 *M*. N_2 . Yellow phosphorus. 40°. Incubation period, 40 min.

Ketonic acid added	Glutamic acid added μ l.	Glutamic acid found μ l.	Glutamic acid disappeared μ l.	% Glutamic acid disappeared
—	1344	1240	104	7.5
Oxaloacetic	1344	654	690	51.5
Pyruvic	1344	688	656	49
α -Ketobutyric	1344	1090	254	19
α -Ketovaleric	1344	1180	164	12
α -Ketocaproic	1344	1190	154	11
Mesoxalic	1344	1105	239	18
Acetopyruvic	1344	1162	182	13.5
Acetoacetic	1344	1192	152	11
Laevulic	1344	1360	—	—

it is seen that pyruvic acid is the most active, α -ketobutyric acid is slightly active, while α -ketovaleric and α -ketohexoxic acids are not appreciably active. These results are in keeping with the findings of the reverse reactions (Table I). Of the α -ketonic dicarboxylic acids, oxaloacetic acid shows an activity of the same order as pyruvic whilst mesoxalic shows a small activity. Acetoacetic, acetopyruvic and laevulic acids are not appreciably active. Laevulic acid appears to inhibit the small anaerobic disappearance of glutamic acid.

All the preceding observations on transamination in pigeon breast muscle can be explained on the assumption that three different enzyme systems are concerned with transamination in this tissue, one each for the three reactions:

Glutamic acid \rightleftharpoons α -ketoglutaric acid

Aspartic acid \rightleftharpoons oxaloacetic acid

Alanine \rightleftharpoons pyruvic acid

The slight activities of *l*(+)-valine and *dl*- α -aminobutyric acid, on the one hand, and α -ketobutyric acid and mesoxalic acid on the other, may be explained by assuming slight affinities of *l*(+)-valine, *dl*- α -aminobutyric and α -ketobutyric acids for the alanine \rightleftharpoons pyruvic acid system, and of mesoxalic for the aspartic \rightleftharpoons oxaloacetic acid system. It is well known that the affinities of other enzyme systems, for example, lactic dehydrogenase, are not strictly limited to one substrate. Homologous compounds may also react but the rate of reaction rapidly falls off as the carbon chain is lengthened. If this holds for the transaminating enzymes, then the failure of the higher homologues of alanine and pyruvic acid to react is easily understood. The fact that α -aminobutyric acid is physiologically a rare compound makes the explanation of its activity difficult on any other basis.

Glutamic acid formation from amino compounds other than α -amino-acids

A number of different amino compounds— β -, δ -, and ϵ -amino-acids, amines and aminopurines—were tested for glutamic acid formation in the presence of

α -ketoglutaric acid, but no significant increase was observed with any of these compounds (Table IV). These findings confirm those of Braunstein & Kritzmann [1938].

Table IV. *Glutamic acid formation from α -ketoglutaric acid and amino compounds other than α -amino acids*

Substrate conc. 0.017 *M*. N_2 . Yellow phosphorus. 40°.

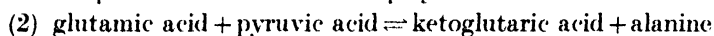
Amino-acid added	Period of incubation min.	Glutamic acid formed	
		Without amino-acid μ l.	With amino-acid μ l.
β -Alanine	30	85	114
δ -Aminovaleric	30	85	114
ϵ -Aminohexaic	30	85	92
<i>dl</i> - β -Aminobutyric	40	130	127
Adenine	40	138	95
Histamine	40	138	118
Putrescine	20	122	125
Spermine	40	122	116
Heptylamine	40	122	92

Rates of formation and disappearance of glutamic acid by transamination

The data in the previous sections are not accurate measurements of the rate of transamination, since in cases where the rate is very rapid, as with *l*(-)-aspartic acid and *l*(+)alanine, the reaction comes to an equilibrium before the end of the incubation period. To determine the rate of transamination shorter periods of incubation were therefore chosen. The data from such experiments are shown in Figs. 1, 2 and 3.

From Fig. 1, it is seen that the initial rates are very rapid for *l*(-)-aspartic acid and *l*(+)alanine. In the case of *dl*- α -aminobutyric acid and *l*(+)valine, the initial rates are also appreciable but the curves show an early plateau, for some unexplained reason. This is not due to a true equilibrium since the plateaux reached from both sides of the reaction lie at different levels (Fig. 2).

Under the experimental conditions employed the reaction:

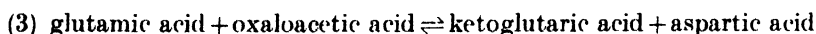


has an equilibrium constant

$$\frac{[\text{glutamic}] \times [\text{pyruvic}]}{[\text{ketoglutaric}] \times [\text{alanine}]}$$

of approximately 1 (see Fig. 2). This is in agreement with the previous finding reported by Braunstein & Kritzmann [1937]. None of the remaining systems shown in Fig. 2 shows any evidence of reaching an equilibrium.

The reaction:



reaches an equilibrium even faster than reaction (2) (Fig. 3). The equilibrium constant of this reaction is of an order similar to that of reaction (3). Owing to some side reactions in which oxaloacetic acid disappears, reaction (3) does not come to a standstill and this complicates the accurate determination of the equilibrium constant.

If the rates for reactions (2) and (3) are expressed as $Q_{\text{glutamic acid}}$,

$$\frac{\mu\text{l. glutamic acid formed}}{\text{mg. dry wt. of tissue} \times \text{hr.}}$$

values of 39 and 44 respectively are obtained. Q_{O_2} values for respiration of pigeon breast muscle under optimum conditions are of the same order.

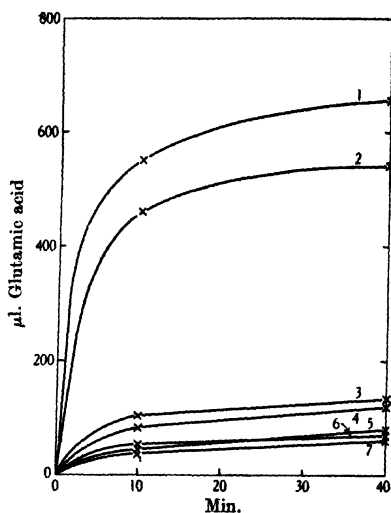


Fig. 1.

Fig. 1. Rate of glutamic acid formation from α -ketoglutaric acid and amino-acids. Substrate conc. $0.017 M$ (for dl -amino-acids, $0.034 M$). N_2 . Yellow phosphorus. 40° . (Corrected for blank.) Curves: 1, $l(-)$ Aspartic acid + α -ketoglutaric acid; 2, $l(+)$ alanine + α -ketoglutaric acid; 3, dl - α -aminobutyric acid + α -ketoglutaric acid; 4, $l(+)$ valine + α -ketoglutaric acid; 5, $l(+)$ phenylalanine + α -ketoglutaric acid; 6, $l(-)$ cysteine + α -ketoglutaric acid; 7, $l(-)$ tyrosine + α -ketoglutaric acid.

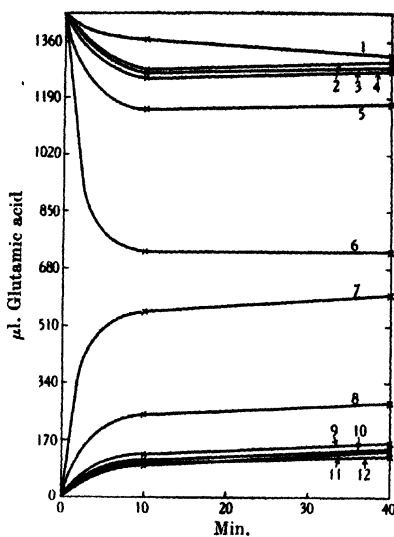


Fig. 2.

Fig. 2. Rate of glutamic acid formation in the presence of α -ketoglutaric acid plus amino-acids, and rate of glutamic acid disappearance in the presence of ketonic acids. Substrate conc. $0.017 M$ (dl -amino-acids, $0.034 M$). N_2 . Yellow phosphorus. 40° . Curves: 1, Glutamic acid; 2, glutamic acid + acetoacetic acid; 3, glutamic acid + α -ketohexonic acid; 4, glutamic acid + α -ketovaleric acid; 5, glutamic acid + α -ketobutyric acid; 6, glutamic acid + pyruvic acid; 7, α -ketoglutaric acid + dl -alanine; 8, α -ketoglutaric acid + dl - α -aminobutyric acid; 9, α -ketoglutaric acid + dl - α -aminovaleric acid; 10, α -ketoglutaric acid + dl - α -aminohexonic acid; 11, α -ketobutyric acid + dl - β -aminobutyric acid; 12, α -ketoglutaric acid.

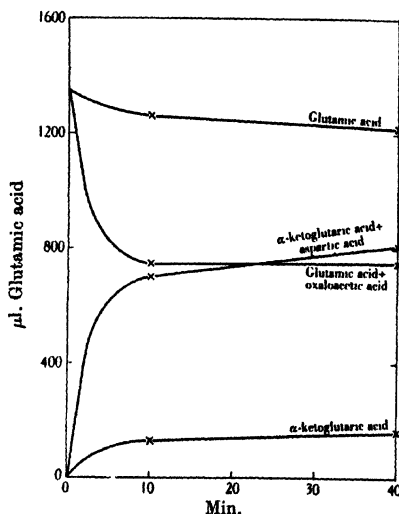


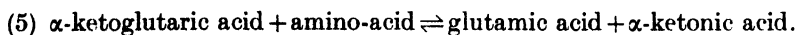
Fig. 3. Rate of glutamic acid formation in the presence of α -ketoglutaric acid plus aspartic acid, and rate of glutamic acid disappearance in the presence of oxaloacetic acid. Substrate conc. $0.017 M$. N_2 . Yellow phosphorus. 40° .

Effect of small amounts of aspartic acid on transamination

The lack of a suitable method for the determination of aspartic acid has so far prevented an investigation of the reaction:



(except when the amino-acid is glutamic acid). However, reaction (4) can be studied in an indirect way by coupling it with reaction (3). Experimentally this means the addition of α -ketoglutaric acid to the system represented by reaction (4). The balance sheet of reactions (3) and (4) is:



Thus it should be possible to detect transamination according to reaction (4) by the addition of α -ketoglutaric acid, aspartic acid and another amino-acid to a muscle suspension. When these three substances are added, reaction (3) takes place and oxaloacetic acid is formed. Oxaloacetic acid would then be expected to react according to reaction (4), and aspartic acid would be regenerated. Since aspartic acid is expected to act in the manner of a catalyst in this reaction, small concentrations of aspartic acid should suffice to bring about an effect. With this in mind previously studied amino-acids were tested with and without the addition of aspartic acid. From the data in Table V it is seen that there is no significant

Table V. *Effect of small concentrations of aspartic acid on transamination from α -ketoglutaric acid and different amino-acids*

Final conc. of α -ketoglutaric acid and amino-acids, 0.016 *M*. Final conc. of aspartic acid, 0.0016 *M*. Substrate added after 5 min. incubation. N_2 . Yellow phosphorus. 40°.

Amino-acid added	Period of incubation with substrate min.	μ l. Glutamic acid formed		Increase in glutamic acid due to aspartic acid μ l.
		Without aspartic acid	With aspartic acid	
—	40	110	194	84
l(+)Alanine	40	520	560	40
l(+)Valine	40	164	218	54
l(-)Phenylalanine	40	140	210	70
l(+)Arginine	40	129	222	93
l(-)Leucine	40	138	241	103

increase in glutamic acid formation from the various amino-acids in the presence of aspartic acid, beyond that of aspartic acid itself. The conclusion may be drawn from these experiments that oxaloacetic acid reacts with no amino-acids other than glutamic acid and alanine. However direct proof for the reaction with alanine has not been produced.

The effect of inhibitors on transamination

The effects of different inhibitors on transamination are listed in Table VI. In these experiments the inhibitors were incubated with the muscle suspension for 15 min. before the substrate was added from the side arm.

Transamination is markedly inhibited by high concentrations of cyanide. As seen from the data the percentage inhibition is roughly proportional to the concentration of cyanide. The effect of high concentrations of cyanide suggests that the inhibition may be due to cyanohydrin formation [Green & Williamson, 1937]; however, the 30 % inhibition by 10^{-3} *M* cyanide indicates that some other basis for the inhibition may exist.

Table VI. *Effect of inhibitors on glutamic acid formation from α -ketoglutaric acid and *l*(+)-alanine and *l*(-)-aspartic acid*

Final substrate conc. 0.015 *M*. Inhibitor incubated with muscle suspension for 15 min. before substrates added. N₂. Yellow phosphorus. 40°.

Inhibitor	Final conc. <i>M</i>	Incubation period min.	Glutamic formed μ l.	Inhibition %
α -Ketoglutaric acid + <i>l</i> (+)-alanine				
—	—	30	576	—
Cyanide	0.05	30	122	79
"	0.01	30	288	50
"	0.001	30	400	30
"	0.0001	30	505	12
Malonate	0.10	30	484	10
"	0.01	30	542	6
Pyrophosphate	0.01	30	534	7
NaF	0.02	30	490	15
Iodoacetate	0.002	30	550	5
Bromoacetate	0.002	30	526	9
As ₂ O ₃	0.01	30	488	15
Octyl alcohol	Sat.	30	498	14
α -Ketoglutaric acid + <i>l</i> (-)-aspartic acid				
—	—	40	592	—
Malonate	0.10	40	436	26
"	0.01	40	580	2

Malonate in concentrations as high as 0.10 *M* has only a slight effect on transamination in the presence of *l*(+)-alanine and α -ketoglutaric acid. Transamination in the presence of *l*(-)-aspartic acid and α -ketoglutaric acid appears to be somewhat more sensitive to 0.10 *M* malonate. However the inhibition at this high concentration is probably non-specific, since at lower concentrations (0.01 *M*) no inhibitory effect is observed with either *l*(+)-alanine or *l*(-)-aspartic acid.

NaF, As₂O₃ and octyl alcohol have small inhibitory effects, while pyrophosphate, iodoacetate and bromoacetate have no appreciable effect on transamination in the presence of *l*(+)-alanine and α -ketoglutaric acid.

The results with As₂O₃ and octyl alcohol are in agreement with those reported by Braunstein & Kritzmann [1937] and Kritzmann [1938]. The results with malonate do not agree with the statement made by Braunstein [1939].

DISCUSSION

It has been pointed out in a previous section of this paper that α -ketoglutaric acid forms glutamic acid when added alone to pigeon breast muscle. Preliminary experiments show that the anaerobic glutamic acid synthesis from ammonium α -ketoglutarate is too small to account for the total glutamic acid formed. This must mean that there are NH₂-donators other than NH₃ preformed in the muscle. Among the amino-acids, *l*(+)-alanine and *l*(-)-aspartic acid are the only ones which could act in this manner. This suggests that these two amino-acids, like glutamic acid [Cohen, 1939], occur free in pigeon breast muscle.

The fact that the three amino-acids which alone are active in transamination are also the only ones which have specific dehydrogenases in muscle [Thunberg, 1920-1; Ahlgren, 1924; Needham, 1930; Euler *et al.* 1938; Dewan, 1938] suggests that transamination depends on the activity of a dehydrogenase during some phase of the reaction. On this basis the limitation of transamination in pigeon breast muscle to those amino-acids which possess dehydrogenases may be understood.

SUMMARY

1. Transamination (Braunstein & Kritzmann) has been studied in pigeon breast muscle. Of 21 different α -amino-acids studied in the presence of α -ketoglutaric acid, *l*(-)-aspartic acid and *l*(+)-alanine are the most active in forming glutamic acid; *dl*- α -aminobutyric acid and *l*(+)-valine are slightly active. None of the remaining amino-acids is appreciably active.

2. Of a series of ketonic acids tested, oxaloacetic and pyruvic acids show the greatest activity in causing the anaerobic disappearance of glutamic acid; α -ketobutyric and mesoxalic acids are slightly active; α -ketovaleric, α -keto-hexoic, acetoacetic and laevulic acids are not appreciably active.

3. The data from these experiments can be explained by assuming the existence of three enzyme systems which are concerned with the following reactions:

Glutamic acid \rightleftharpoons α -ketoglutaric acid

Aspartic acid \rightleftharpoons oxaloacetic acid

Alanine \rightleftharpoons pyruvic acid

By combinations of these three systems the following reactions may be brought about:

(1) α -Ketoglutaric acid + alanine \rightleftharpoons glutamic acid + pyruvic acid.

(2) α -Ketoglutaric acid + aspartic acid \rightleftharpoons glutamic acid + oxaloacetic acid.

(3) Oxaloacetic acid + alanine \rightleftharpoons aspartic acid + pyruvic acid.

The occurrence of reaction (3) in pigeon breast muscle has not yet been conclusively demonstrated. The slight activity of certain homologues can be explained by a slight affinity for one of these enzyme systems.

4. Transamination in pigeon breast muscle is inhibited by high concentrations of cyanide. NaF, As₂O₃ and octyl alcohol have small inhibitory effects. Pyrophosphate, malonate, iodoacetate and bromoacetate have no appreciable effect.

5. Of a number of different amino compounds tested other than α -amino-acids, none has been found to be active in transamination.

6. Certain discrepancies between the results of Braunstein & Kritzmann [1938] and those reported here are discussed. The failure of these authors to use a suitable control in their experiments explains their statement that all α -amino-acids of the *l*-series are active in transamination in pigeon breast muscle.

I wish to express my thanks to Dr H. A. Krebs for his constant advice and help throughout this study.

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CLXXXIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LXIII. ITACONIC ACID, A METABOLIC PRODUCT OF A STRAIN OF *ASPERGILLUS TERREUS* THOM

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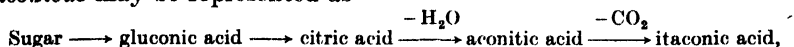
(Received 24 July 1939)

THE study of the metabolic processes of different cinnamon to brown strains in the *Aspergillus terreus* Thom series has formed the subject of a number of communications from these laboratories. Raistrick & Smith [1935] reported that two strains out of five grown on Czapek-Dox solution gave a hitherto undescribed mould metabolic product—terrein, $C_8H_{10}O_3$ —which was shown by Clutterbuck *et al.* [1937, 2] to be 4-propenyl-2-hydroxy-3:5-oxidocyclopentane-1-one. One of these two strains gave, in addition to terrein, considerable quantities of citrinin, $C_{13}H_{14}O_6$, a crystalline yellow colouring matter previously reported as a metabolic product of *Penicillium citrinum* Thom by Hetherington & Raistrick [1931]. Of the other three strains of *A. terreus* one gave succinic acid, another oxalic acid, while the third strain gave a mixture of these two acids. In a later communication Raistrick & Smith [1936] showed that one of the five strains previously examined by them, which produced terrein also, metabolized almost the whole of the chloride supplied as KCl in the Czapek-Dox solution and gave rise to two new chlorine-containing metabolic products, geodin $C_{17}H_{12}O_7Cl_2$ and erdin $C_{16}H_{10}O_7Cl_2$, the molecular constitutions of which have been investigated [Clutterbuck *et al.* 1937, 1; Calam *et al.* 1939].

The main purpose of the present communication is to give the results of an investigation of the metabolic products of a recently isolated but indubitable strain of *A. terreus*, L.S.H.T.M. Cat. No. Am. 1. This strain, when grown on Czapek-Dox solution, gives none of the metabolic products described above, nor does it utilize to any appreciable extent any of the KCl present in the medium, but instead it produces considerable quantities of itaconic acid.

This acid has only once previously been reported as a mould metabolic product, nor indeed, so far as we are aware, has it been reported from any other biological source. Kinoshita [1931, 1] described a new species of *Aspergillus* *A. itaconicus*, which he isolated from the juice of salted plums. This green species, which is quite distinct from *A. terreus*, is exceptional in that it will only grow well on media of high osmotic pressures such as concentrated sugar solutions and produces very large amounts of itaconic acid on media containing KNO_3 as nitrogen source and 25% of sucrose. Smaller yields of itaconic acid are formed on 25% glucose solutions [Kinoshita, 1931, 2]. When *A. itaconicus* was grown on sucrose or glucose solutions containing $CaCO_3$, appreciable amounts of calcium citrate and calcium gluconate were formed in addition to calcium itaconate, and

Kinoshita therefore concluded that the course of the metabolism of sugar by *A. itaconicus* may be represented as



although no aconitic acid could be detected in the metabolism solution.

Our experiments, while giving no clue as to the stages intermediate between glucose and itaconic acid, offer no support for the view that citric acid is an intermediate product in the formation of itaconic acid by our strain of *A. terreus*. Thus in a large scale experiment, with an incubation period of 25 days and using the ordinary Czapek-Dox 5% glucose medium, the amounts of itaconic and citric acids present were estimated at intervals of a few days. The amounts of itaconic acid increased steadily from the fifth day of incubation to the end of the incubation period but at no time during the whole of the experiment could any citric acid be detected by the pentabromoacetone method. At the end of the incubation period, when all the glucose had been metabolized, the metabolism solution was drained as carefully as possible from the mycelium and was replaced in separate flasks by the following series of substrates: (1) Czapek-Dox salts solution without glucose or NaNO_3 . (2) Czapek-Dox salts solution as in (1) and containing in separate flasks 1% of the following: glucose, citric acid, malic acid, pyruvic acid, acetic acid, malic + acetic acids, pyruvic + acetic acids. Glucose was the only substrate which gave yields of itaconic acid which were substantially larger than those obtained in the Czapek-Dox salts solution control.

Finally, the metabolisms of five other strains of *A. terreus*, three of which have not been examined previously, were investigated. None of these strains utilized any appreciable amount of the KCl supplied in the medium, three strains produced succinic acid, one oxalic acid and one fumaric acid. Amorphous precipitates were produced on acidification of the metabolism solution from all strains at the end of the incubation period, often in considerable amounts, but in no case could any crystalline substance be isolated from these precipitates.

EXPERIMENTAL

History of culture used for production of itaconic acid

The strain of *Aspergillus terreus* used, L.S.H.T.M. Cat. No. Am. 1, was isolated in 1935 by Mr G. Smith from American cotton yarn. It is without question a strain of *A. terreus* and forms typical, velvety, cinnamon-coloured colonies on Czapek-Dox agar.

Cultural conditions

The medium used was a Czapek-Dox solution of the following composition: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l. 350 ml. quantities of this solution were placed in a number of 1-litre conical flasks plugged with cotton wool which were sterilized, inoculated with a spore suspension of *A. terreus*, Am. 1, and incubated in the dark at 24°.

Exp. 1

In this experiment 97 flasks were used. At intervals during the incubation period single flasks were taken off, the contents filtered, and the following estimations were carried out on the filtrate.

(1) Residual glucose by polarimeter.

(2) Chlorine in solution as inorganic chloride by the method described by Raistrick & Smith [1936].

(3) Total chlorine in solution. 50 ml. of the filtrate were made alkaline with a few drops of NaOH and evaporated to dryness in a nickel crucible. The residue was fused with a mixture of NaOH (10 g.) and Na_2O_2 (5 g.). The cooled melt was acidified with HNO_3 and the chloride estimated by titration with $N/10$ AgNO_3 by the Volhard method.

The results are given in Table I and prove that this strain of *A. terreus*, unlike strain No. 45 [Raistrick & Smith, 1936] which gives the chlorine-containing substances geodin and erdin, produces little, if any, water-soluble organic compounds containing chlorine.

Table I

Incubation period days	Residual glucose %	Inorganic chloride as KCl %	Total chlorine as KCl %
0	5.00	0.050	0.050
9	3.50	0.052	0.052
16	1.53	0.052	0.053
23	0.39	0.051	0.055
30	0.00	0.046	0.048
36	0.00	0.052	0.052
Av. of 93 flasks			

At the end of the incubation period (36 days) the metabolism solution in the remaining 93 flasks was separated by filtration and the mycelium was washed with water, dried and weighed (wt. 440 g. = 4.73 g. per flask). The yellow metabolism solution and washings were combined, acidified to Congo red with conc. HCl, kept for 2 days and filtered from a yellow amorphous solid (3.5 g.). The filtrate was neutralized to litmus with NaOH, evaporated *in vacuo* to 700 ml., and a small amount of amorphous material was separated by centrifuging. On acidifying the clear supernatant liquid with HCl, itaconic acid separated as a mass of dark brown crystals, wt. 62.5 g. The itaconic acid remaining in solution (33 g.) was extracted with ether in a continuous extractor as almost colourless crystals. The combined crops (95.5 g. \equiv 5.9% of glucose metabolized) were purified by crystallizing from ethyl acetate and itaconic acid was finally obtained as large colourless prisms, m.p. 164–167°, not depressed on admixture with an authentic specimen. (Found: C, 46.15, 46.20; H, 4.81, 4.99; equiv. by titration, 65.6. Calc. for $\text{C}_5\text{H}_4\text{O}_4$: C, 46.15; H, 4.65%; equiv. as a dibasic acid, 65.0.) 1.192 g. of the acid, shaken in aqueous solution with a palladium-charcoal catalyst, absorbed 204 ml. of hydrogen at N.T.P. (calc. 205 ml.). On ether extraction of the solution 0.9 g. of methylsuccinic acid was obtained, m.p. 108–111°, (lit. 111–112°). (Found: equiv. by titration, 65.6. Calc. for $\text{C}_5\text{H}_8\text{O}_4$ titrating as a dibasic acid, 66.0.)

Exp. 2

In this experiment, in which 108 flasks each containing 350 ml. of Czapek-Dox solution were used, a more extensive examination was made of the course of metabolism. Individual flasks were taken off at frequent intervals and the metabolism solution was separated by filtration from the mycelium which was washed with water, dried *in vacuo* and weighed. The filtrate and washings were made up to 500 ml. The following estimations were carried out on aliquot portions and the results are given in Table II in which all figures are calculated as for 1 flask.

- (1) Residual glucose by polarimeter.
- (2) Residual glucose by the Shaffer-Hartmann method.
- (3) pH colorimetrically.
- (4) Acidity by titration with $N/10$ NaOH to phenolphthalein.

(5) Permanganate reduction. This somewhat empirical estimation was carried out as follows: $N/10$ $KMnO_4$ was gradually added to 20 ml. of the metabolism solution, acidified with 10 ml. of $2N$ H_2SO_4 . The end-point, which was not very well defined, was taken when decoloration of the permanganate ceased to be immediate.

(6) Bromine absorption by the Koppeschaar method [1876].

(7) Itaconic acid. 100 ml. of the metabolism solution were evaporated *in vacuo* to 5–10 ml., acidified with HCl and centrifuged until clear. The supernatant was extracted continuously with ether and the residue obtained on removing the solvent was sublimed in a high vacuum at 100 – 110° . The sublimate, consisting of almost pure itaconic acid, was weighed and its identity proved by crystallization from ethyl acetate-light petroleum, M.P. and mixed M.P.

(8) Citric acid. The method used is described in Allen, *Commercial Organic Analysis* (Churchill, 1924), 1, pp. 751–2. The citric acid was isolated as the barium salt which was converted into pentabromoacetone and this was collected and weighed. Tests on artificial metabolism solutions, containing glucose and mixtures of various organic acids, including itaconic acid, showed that the method is accurate and specific for citric acid.

Table II

Incubation period days	Mycelium g.	Residual glucose		pH	Acidity		Br absorption		Itaconic acid isolated mg.	Citric acid estimated mg.
		Polarimeter %	Shaffer-Hartmann %		$N/10$ $NaOH$ ml.	$KMnO_4$ $N/10$ ml.	As Br mg.	As itaconic acid mg.		
0	0	3.51	3.46	4.2	26.4	0.0	60	0	—	—
5	0.87	3.82	3.02	6.3	20.8	17.5	200	113	—	—
8	1.67	2.58	2.40	6.4	26.7	39.0	230	138	—	—
11	2.94	1.70	1.60	6.5	14.5	80.0	375	256	—	Nil
15	4.27	0.73	0.79	6.6	8.2	102.5	530	382	75	Nil
19	5.50	0.24	0.23	5.2	48.6	222.5	960	730	615	Nil
22	5.35	0.11	0.16	5.9	16.1	191.3	790	594	400	Nil
25	5.32	0.04	0.08	5.2	44.7	200.0	990	755	707	Nil

After 25 days' incubation the contents of 100 flasks were filtered and the filtrate and mycelium washings were evaporated *in vacuo* at 40 – 50° to a volume of about 1400 ml. The concentrated solution was made acid to Congo red with conc. HCl (100 ml.) diluted to 1800 ml. with water and kept overnight. The precipitate formed was difficult to separate but was removed by filtration through kieselguhr. The clear filtrate was extracted with ether in a continuous extractor and gave crude itaconic acid (70.7 g. $\equiv 4.0\%$ of glucose metabolized), which was purified by crystallization from ethyl acetate-light petroleum. A portion was converted into the phenacyl ester [Rather & Reid, 1919] which after recrystallizing from aqueous methanol melted at 78 – 80° ; the M.P. was not depressed on admixture with a specimen of the phenacyl ester of authentic itaconic acid, which also melted at 78 – 80° . Rather & Reid give the M.P. as 79.5° .

Formation of itaconic acid on different substrates

At the end of the incubation period (25 days) in exp. 2, 24 flasks, chosen at random, were divided into 8 groups of 3 flasks each. The metabolism solution was carefully drained off, so as to disturb the mycelium as little as possible, and was used along with the metabolism solution and mycelium washings from the remaining 76 flasks for the isolation of itaconic acid, as described above. Into each flask, in each group of 3 flasks, were then poured 350 ml. of each of the

following solutions, previously sterilized, the operation being carried out in such a way as to leave the mycelium, as far as possible, floating on the surface of the liquid. (1) Czapek-Dox solution from which was omitted the glucose and sodium nitrate, i.e. KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l. (2) The same solution of salts as in (1) together with (a) 1 % glucose; (b) 1 % citric acid; (c) 1 % malic acid; (d) 1 % pyruvic acid; (e) 1 % acetic acid; (f) 0.66 % malic acid + 0.33 % acetic acid; (g) 0.6 % pyruvic acid + 0.4 % acetic acid. Solutions 2 (b)–2 (g) were brought to pH 4–5 by the addition of aqueous NaOH before sterilization. After incubation for 7 days at 24° the metabolism solution was separated by filtration, the mycelium pressed, dried and weighed. The following estimations were then carried out on the combined metabolism solution and pressings from the mycelium: (1) pH; (2) itaconic acid. The latter was isolated and weighed as described on p. 1491. (3) Residual substrate: (a) glucose—by polarimeter and Shaffer-Hartmann method; (b) citric acid—by pentabromoacetone method; (c) pyruvic acid—by precipitation and weighing as the 2:4-dinitrophenylhydrazone; (d) acetic acid—by distillation after acidification with phosphoric acid, and titration of the distillate. The residual malic acid was not estimated. The results obtained are given in Table III in which all results are expressed as for 1 flask.

Table III

Substrate	Substrate supplied g.	Substrate utilized g.	pH at		Mycelium g.	Itaconic acid isolated g.	Excess itaconic acid formed over Czapek-Dox salt control g.
			Start	Finish			
Glucose, 1 %	3.50	3.20	5.0	4.6	5.88	0.437	0.378
Citric acid, 1 %	3.50	2.20	4.4	5.0	5.46	0.095	0.036
Malic acid, 1 %	3.50	—	4.3	6.0	5.18	0.122	0.063
Pyruvic acid, 1 %	3.50	1.23	4.2	7.1	5.13	0.066	0.007
Acetic acid, 1 %	3.50	0.25	4.2	4.9	4.55	0.047	—
Malic acid, 0.66 %	2.34	0.60	4.2	4.8	4.71	0.063	0.004
Acetic acid, 0.33 %	1.17						
Pyruvic acid, 0.60 %	2.10	0.42	4.2	6.0	4.35	0.050	—
Acetic acid, 0.40 %	1.40	0.30	—	—	—	—	—
Czapek salts	—	—	5.0	5.8	5.13	0.059	—

The results prove that with pyruvic acid, acetic acid and mixtures of malic acid and acetic acid and of pyruvic acid and acetic acid no itaconic acid is formed, while with citric acid or malic acid the amounts of itaconic acid formed are very small compared with the amounts formed from glucose, being about 10 % of the yield from glucose in the case of citric acid and 17 % in the case of malic acid. The yield of itaconic acid obtained from glucose was 11.8 % of the glucose metabolized.

The metabolism of other strains of Aspergillus terreus

History of strains used. Relevant details of the five strains of *A. terreus* used in this part of the work are given in Table IV.

Table IV

Cultures on Czapek-Dox agar

Catalogue no.	Source	Isolated by	Surface colour	Reverse colour	Texture
No. 3	Mildewed cloth	G. Smith, 1926	Cinnamon	Deep yellow	Smooth velvety
No. 37	Egyptian cotton	G. Smith, 1926	Cinnamon	Greenish yellow	Smooth velvety
Am. 2	American cotton	G. Smith, 1935	Cinnamon	Greenish yellow	Slightly floccose
Ac. 107	Laboratory contaminant at Ardeer	J. H. V. Charles, 1926	Cinnamon	Reddish brown	Slightly floccose
E. M. J.	Binding of old vol. of <i>Edin. Med. Journal</i>	H. H. Stroud, 1938	Sand brown	Yellowish brown	Smooth velvety

Table V

<i>A. terreus</i> Cat. No.	Incu- bation period days	No. of flasks	Residual glucose per flask %	My- celium per flask g.	Total Cl as KCl %	Inorganic Cl as KCl %	Acid precipitate	Ether extract I	Neutral precipitate	Ether extract II	Ether extract III
Unown	—	—	5.00	—	0.050	0.050	—	—	—	—	—
No. 3	29	97	0.15	5.12	0.043	0.043	Not weighed	13.0 g. brown glass	17 g. brown solid giving 7 g. viscous oil on ether extraction	Small amount of light yellow oil	Brown oil, 11.8 g., giving 2.25 g. oxalic acid
No. 37	38	89	0.05	5.48	0.049	0.048	23.5 g.	18.2 g. dark brown glass	Phosphates only	0.95 g. brown glass	Brown sticky semi-solid, 20.25 g., giving 3.35 g. fumaric acid
Am 2	34	90	0.14	2.71	0.044	0.044	1.2 g. black floccules	Not extracted	6.65 g. black solid, not extracted	1.35 g. black tar	Black crystals, 8.2 g., gave 3.0 g. succinic acid
Ac 107	36	94	0.00	3.31	0.052	0.048	5.9 g. black solid	Not extracted	Phosphates only	Viscous oil, 0.5 g.	Oil and crystals, 3.9 g., giving 0.72 g. succinic acid
E. M. J.	37	93	0.24	5.05	0.058	0.051	68 g. solid	19.1 g. brown glass	Phosphates only	Brown viscous oil, 0.79 g.	Brown glass and crystals, 14.9 g.; 0.9 g. succinic acid
Am 1 (exp. 1)	36	93	0.00	4.73	0.052	0.052	Yellow solid, 3.5 g.	Not extracted	22.7 g. amorphous brown solid, not extracted	Not extracted	Itaconic acid, 95.5 g.

Cultural conditions and examination of metabolic products. 100 one-litre flasks each containing 350 ml. of Czapek-Dox 5 % glucose solution were sterilized and sown with a spore suspension of the strain of *A. terreus* under investigation, and were then incubated at 24°. At intervals of a few days the residual glucose, total chlorine in solution and inorganic chloride in solution were estimated. However, since none of the strains investigated metabolized any appreciable amount of the chloride present, only the figures at the end of the incubation period are given in Table V. When the glucose had been almost completely metabolized the flasks were removed from the incubator, the mycelium separated by filtration, washed, dried and weighed. The metabolism solution was now made acid to Congo red with conc. HCl and the amorphous precipitate was filtered off (Acid precipitate, col. 8, Table V). In those cases (Strains Nos. 3 and 37 and E.M.J.) where filtration was difficult a weighed amount of kieselguhr was added and the dried kieselguhr and precipitate were exhaustively extracted with ether in a Soxhlet apparatus. Removal of ether gave ether extract I (col. 9). In all cases the acid filtrate was made neutral to litmus with caustic soda and evaporated *in vacuo* at 40–50° to about 1 l. The material which separated and consisted in most cases of inorganic phosphates was filtered off and dried (Neutral precipitate, col. 10). The neutral filtrate was then extracted with ether giving ether extract II (col. 11) and was finally acidified to Congo red and re-extracted with ether giving ether extract III (col. 12). The corresponding figures for strain Am. 1 are added to Table V for comparison.

It is obvious from the results given in Table V that, since none of the six strains examined metabolizes inorganic chloride to any appreciable extent, none of them produces the chlorine-containing substances geodin or erdin. This conclusion was confirmed by tests for chlorine on the acid precipitate and on ether extracts I, II and III. In all cases a negative result was obtained.

No crystalline substance could be isolated, in any instance, either from the acid precipitate or from ether extracts I or II. Ether extract I is acidic in nature and contains alkoxyl groups. Thus ether extract I from strain No. 3 had an equivalent, by titration, of 405 and a methoxyl content, by the Zeisel method, of 6.9 %; while the corresponding figures for strain No. 37 were 458 and 8.9 % and those for strain E.M.J. were 402 and 8.4 %.

The only crystalline metabolic products obtained from any of the strains examined were separated from ether extract III, and with each strain a relatively simple organic acid was isolated from this fraction. The variable nature of these acids, from strain to strain, is of some interest from the point of view of relationship between morphological characteristics and biochemical activities within the boundaries of a mycological "series" of strains of a species of fungus. Thus of the six strains examined one strain gave itaconic acid, one strain oxalic acid, three strains succinic acid and one strain, No. 37, fumaric acid. This strain had been examined previously by Raistrick & Smith [1935] who found that it then gave appreciable amounts of succinic acid and traces of oxalic acid but no fumaric acid was detected.

SUMMARY

It has been shown that itaconic acid is a metabolic product of an indubitable strain of *Aspergillus terreus* Thom when this organism is grown on Czapek-Dox 5 % glucose solution. The evidence presented does not support the view that, with this mould, citric acid is an intermediate stage in the formation of itaconic acid from glucose. Five other strains of *A. terreus* were also grown on Czapek-Dox 5 % glucose solution. None of them produced itaconic acid, three produced succinic acid, one fumaric acid and one oxalic acid.

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CLXXXIV. OBSERVATIONS ON THE POLY-SACCHARIDE COMPLEX PRESENT IN SERUM PROTEINS AND ON THE EFFECT OF PEPSIN ON SERUM PROTEIN FRACTIONS

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It was shown in a previous paper [Hewitt, 1938] that fractional precipitation with acid acetone following peptic digestion of various proteins afforded a convenient method of separating carbohydrate-rich peptones. The use of proteolytic enzymes to separate the carbohydrate component in proteins has been described by a number of workers [Rimington, 1929; Iseki, 1934; Neuberger, 1938], but the subsequent fractionation process is in general lengthy or involves somewhat drastic chemical treatment so that repetitions of the fractionation process become cumbersome and it is difficult to ensure that the final product has suffered no chemical modification during the manipulations. The relatively mild procedure involved in the acid acetone precipitation and the extreme rapidity of the process rendered it worthy of investigation as a method of isolating the polysaccharide residues from serum proteins, amongst which seroglycoid and globoglycoid have been shown to be particularly rich in "bound carbohydrate" [Hewitt, 1934; 1936; 1937; 1938]. Among other points requiring investigation was the question of the initial products of the attack of enzymes on the protein fractions.

Experimental

The protein fractions used were obtained from horse serum. Globoglycoid was prepared from either the globulin fraction precipitated between the limits 45 and 55 % saturation with ammonium sulphate or from the albumin fraction as previously described [Hewitt, 1938]. Seroglycoid was precipitated from the mother liquor after successive removal of globulin fractions and crystalalbumin by $(\text{NH}_4)_2\text{SO}_4$ [Hewitt, 1936; 1937].

Carbohydrate determinations were carried out using the orcinol method [Tillmans & Philippi, 1929; Sørensen & Haugaard, 1933; Hewitt, 1934], colorimetric comparisons being made photoelectrically in most cases. Results are quoted in terms of galactose-mannose-hexosamine.

Fractionation experiments

Peptic digests of seroglycoid, globoglycoid and ovalbumin at pH 1.5 were treated with acetone (5.5 vol.) and after removing the precipitate by centrifuging a second precipitate was produced by adjusting the pH to 4.8. After centrifuging the supernatant fluid was evaporated to dryness. The properties of the fractions are summarized in Table I.

It will be seen that the bulk of the carbohydrate is to be found in the acid acetone precipitate. A more detailed fractionation on a larger scale was now carried out as follows. A solution of globoglycoid was brought to pH 1 by

Table I. *Acetone fractionation of peptic digests*

Fraction	Seroglycoid		Globoglycoid		Ovalbumin	
	Weight mg.	Carbo- hydrate %	Weight mg.	Carbo- hydrate %	Weight mg.	Carbo- hydrate %
Acid acetone	91	20.0	33	24.6	50	18.6
Neutralized acetone	248	1.8	58	3.0	133	0.3
Evaporated residue	96	0	49	0	79	1.3

addition of HCl, 0.3 % of pepsin was added and the mixture was incubated at 50° for 22 hr. Acetone (4 vol.) was added, the precipitate was removed by centrifuging, and more acetone (4 vol.) was added. Again the precipitate was centrifuged down and a further fraction was obtained by neutralizing the supernatant fluid with NaOH. The precipitates were washed with acetone and ether and dried *in vacuo*. The composition of the fractions is summarized in Table II.

Table II

Fraction	Weight g.	Carbohydrate content %
80 % acid acetone	0.44	39
90 % acid acetone	0.88	9.7
Neutralized acetone	1.61	1.0

It is clear that the bulk of the polysaccharide-containing material is precipitated by acid acetone. An experiment, using tryptic digestion of heated globoglycoid in place of peptic digestion, was conducted as follows. A globoglycoid solution was heated in a boiling water bath and cooled, the pH was adjusted to 8, trypsin was added and the mixture was incubated at 45° for 24 hr. For the first 8 hr. small additions of trypsin, together with NaHCO₃ to maintain the pH at 8, were made at 2 hr. intervals. After incubation the digest was acidified to pH 1 by addition of HCl, acetone (4 vol.) was added and the precipitate was collected and dried *in vacuo* after dehydration with acetone and ether. The carbohydrate content (25 %) was rather lower than in comparable experiments with pepsin, when figures between 27 and 39 % were observed. On the basis of these experiments a method of preparing the polysaccharides from serum proteins was worked out.

Preparation of polysaccharides

Proteins (globoglycoid and seroglycoid) were first subjected to peptic digestion at pH 1 for 24 hr. at 50°. The carbohydrate-rich fraction of peptone was then precipitated by addition of acetone (4 or 5 vol.) to the acid solution, centrifuged down, washed with acetone and ether and dried *in vacuo*. The dried powder was then dissolved in water and subjected to tryptic digestion. The solution was made alkaline by addition of NaHCO₃, trypsin was added and the mixture was incubated at 45° for 24 hr., further small quantities of trypsin and NaHCO₃ being added at 2 hr. intervals for the first 8 hr. of incubation. In all about 0.2 % of trypsin was added. After incubation the trypsin digest was acidified to pH 1 with HCl, acetone (5 vol.) was added and the precipitate was collected and dried as before. Following the peptic digestion two or three successive tryptic digestions, conducted under similar conditions, were used in the preparation of each specimen of polysaccharide. Typical experiments are summarized in Table III.

Table III. *Properties of typical digest products*

Digest	Globoglycoid			Seroglycoid		
	Weight g.	Carbo- hydrate %	N content %	Weight g.	Carbo- hydrate %	N content %
Pepsin	2.7	30	8.2	5.3	20	10.5
1st Trypsin	1.45	44	5.9	2.2	37.5	7.3
2nd Trypsin	1.0	53	4.8	1.4	43.5	5.9
3rd Trypsin	—	—	—	1.15	51	5.3

It appeared that seroglycoid was more resistant to digestion than globoglycoid although in other experiments the differences were less than those shown in Table III.

Properties of the polysaccharides

When first precipitated with acid acetone from aqueous solution the polysaccharides were in the form of sticky gums, but when treated with acetone and ether they were obtained as fine white powders. These were readily soluble in water, giving clear, colourless solutions. Like the polysaccharide from *B. dysenteriae* [Morgan, 1937] the serum protein polysaccharides were soluble in ethylene glycol but were precipitated by addition of acetone. The polysaccharides were not dissolved by glacial acetic acid. The carbohydrate contents, as determined by the colorimetric orcinol method, ranged from 51 to 55 %, and the N content from 4.8 to 5.3 %. The Molisch reaction was strongly positive at a dilution of 1 : 1,000,000. The reactions with the biuret reagent, the phenol reagent and with phosphotungstic acid were all negative or very faintly positive. The colorimetric reaction for cystine with the uric acid reagent was very faintly positive after acid hydrolysis. After acid hydrolysis the polysaccharides possessed strong reducing properties as will be seen later, but before hydrolysis the trace of reducing power that could be detected was due to the presence of traces of amino-acids as described in a previous communication [Hewitt, 1938].

Samples of the polysaccharides were hydrolysed by heating for 4 hr. in a boiling water bath with 2.5 *N* HCl, or with 1.5 *N* *p*-toluenesulphonic acid for 2 hr. The reducing power was determined by a modified Hagedorn-Jensen method [Hanes, 1929; Hulme & Narain, 1931; Hewitt, 1938] and hexosamine was determined by a colorimetric method [Hewitt, 1938] based on that of Zuckerkandl & Messiner-Klebermass [1931], Elson & Morgan [1933], Nilsson [1936], and Meyer *et al.* [1936]. Acetyl determinations were carried out after hydrolysis with *p*-toluene sulphonic acid using the technique of Elek & Harte [1936]. Considerable humin formation occurred during the hydrolyses. The results are summarized in Table IV.

Table IV. *Analysis of hydrolysed polysaccharides*

Source	Method of hydrolysis	Carbo- hydrate %	Hexos- amine %	Acetyl %
Globoglycoid	2.5 <i>N</i> HCl	55	22	—
"	1.5 <i>N</i> MePhSO ₃ H	55	17	4.4
"	2.5 <i>N</i> HCl	61	23	—
Seroglycoid	2.5 <i>N</i> HCl	51	18	—
"	1.5 <i>N</i> MePhSO ₃ H	52	16	5.1
"	2.5 <i>N</i> HCl	—	20	—
"	2.5 <i>N</i> HCl	56	23	—

The hydrolysates when heated with orcinol and sulphuric acid developed a colour similar to that given by an equimolecular mixture of galactose and mannose. Assuming, therefore, that the polysaccharide has the composition galactose-mannose-acetylhexosamine the analytical figures for a total carbohydrate content of 55 % should be hexosamine 18.3 % and acetyl 4.4 %. It will be seen that the above figures are in reasonable agreement with the theoretical expectation.

The amount of "diamino-nitrogen" precipitable by phosphotungstic acid was determined after hydrolysis with concentrated HCl for 4 hr. on an electric sand bath in a flask fitted with an air condenser. Considerable discoloration due to humin formation was again observed. The HCl was removed by evaporation *in vacuo*, the residue was dissolved in water and the humin filtered off. The solution was made alkaline and NH_3 was removed by gently warming the solution *in vacuo*. The solution was acidified with H_2SO_4 and boiled and the diamino-N was precipitated by addition of 5 % of phosphotungstic acid in the presence of 5 % H_2SO_4 to the boiling solution. After standing overnight in the ice-chest, the precipitate was centrifuged down, washed with ice-cold phosphotungstic acid solution and dissolved in dilute alkali for N determinations. The diamino-N accounted for about 21 % of the total N in the polysaccharide. Although some colour was developed when the diazo-reagent was added to the dissolved phosphotungstic acid precipitate, the Knoop bromine reaction was extremely weak so that it would appear that the content of histidine was not great. It is interesting to note that in the case of egg albumin polysaccharide Neuberger [1938] was unable to account for that portion of the phosphotungstic acid precipitate which was not ammonia.

The optical rotatory powers of the polysaccharides were examined and they were found to be laevorotatory, but after acid hydrolysis they became dextro-rotatory as shown in Table V.

Table V. *Specific rotations of polysaccharides*

Polysaccharide from	$[\alpha]_{5161}$	
	Before hydrolysis	After hydrolysis
Globoglycoid	-20°	+32°
"	—	+37°
Seroglycoid	-20	+34°
"	-17°	+31°

Ultra-violet absorption spectra

In view of the value of ultra-violet absorption spectra in differentiating proteins according to their content of certain aromatic amino-acids [Dhére, 1909; Stenström & Reinhard, 1925; Smith, 1929; Hewitt, 1929; Coulter *et al.* 1936; Holiday, 1936] the absorption spectra of the polysaccharides and of other digest products were examined using a Hilger quartz spectrograph and photometer. In Figs. 1 and 2 are seen the absorption spectra in alkaline solution during successive stages in the digestion of globoglycoid and seroglycoid. The gradual diminution of the absorption in the neighbourhood of wave-lengths 2800 and 2900 Å is seen, showing that tyrosine and tryptophan are split off at a relatively early stage in the digestion, leaving the polysaccharide almost entirely free from these amino-acids. The absorption spectra of the polysaccharides themselves under different conditions are shown in Fig. 3, plotted on a different scale, the solutions examined being four times more concentrated than

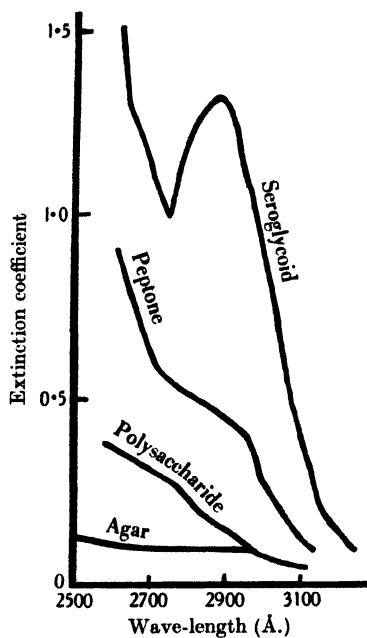


Fig. 1.

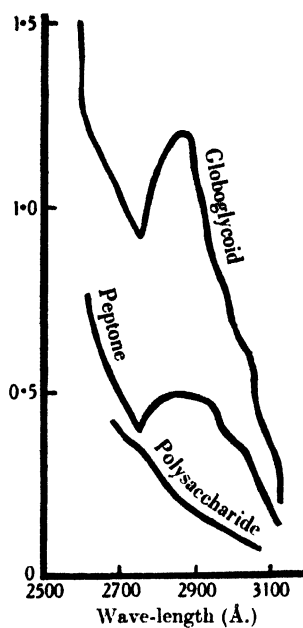


Fig. 2.

Fig. 1. Ultra-violet absorption spectra during successive stages of seroglycoid digestion (1 cm. layer; 0.1%).

Fig. 2. Ultra-violet absorption spectra of globoglycoid and its digestion products (0.1%; 1 cm. layers).

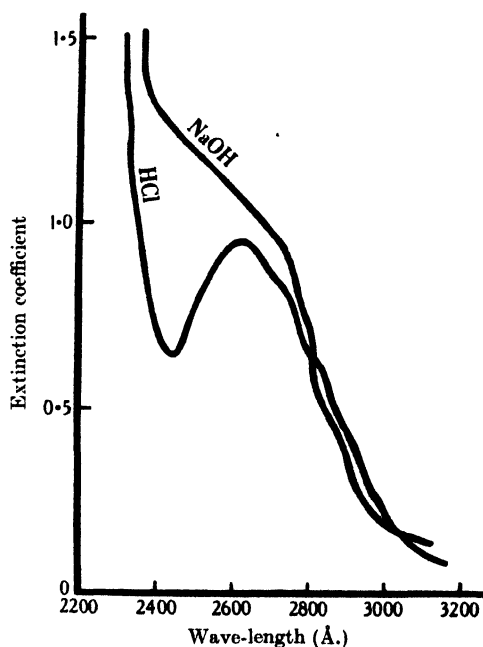


Fig. 3. Ultra-violet absorption spectra of globoglycoid polysaccharide in 0.1 N NaOH and 0.1 N HCl (0.4% solution; 1 cm. layers).

those in Figs. 1 and 2. It will be seen that an absorption band in the neighbourhood of 2650 Å. is clearly seen in acid solution but this is apparently obscured by an increase in the general absorption in alkaline solution. The position of the peak of the band is fairly close to that of phenylalanine but is well removed from that of tryptophan or tyrosine. Polysaccharides like agar show no absorption bands in this region of the spectrum.

Trypsin and pepsin polysaccharides

The method outlined above for isolating polysaccharides was applied to trypsin and pepsin themselves. No appreciable amount of polysaccharide was obtained from commercial trypsin, but from commercial pepsin a small amount of polysaccharide was isolated. Pepsin (10 g.) in aqueous solution was allowed to

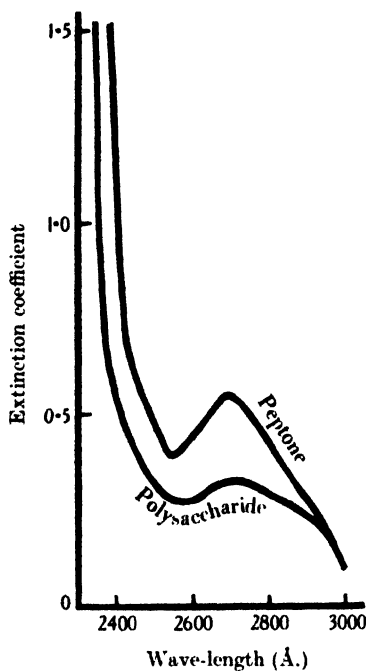


Fig. 4.

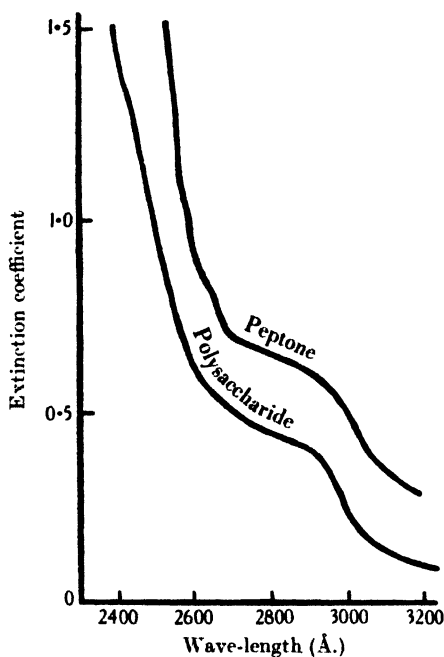


Fig. 5.

Fig. 4. Pepsin autolysis products; ultra-violet absorption spectra in 0.1 *N* HCl (0.4 % solutions; 1 cm. layers).

Fig. 5. Ultra-violet absorption spectra of pepsin; autolysis products in 0.1 *N* NaOH (0.4 % solutions; 1 cm. layers).

autolyse at pH 1.1 for 18 hr. at 45°. Addition of acetone (2.5 vol.) produced a precipitate which, when dehydrated, was a white powder weighing 0.2 g., containing 54 % of carbohydrate and having a rotatory power $[\alpha]_{5461}$ of -35° . Addition of more acetone (2 vol.) to the mother liquors yielded a peptone (1.3 g.) containing 19 % of carbohydrate and 11 % of N with $[\alpha]_{5461} -105^\circ$. The ultra-violet absorption spectra of these products are shown in Figs. 4 and 5 and it will be seen that the general characteristics of the serum protein polysaccharides are encountered again, with the absorption band visible in acid solution, but obscured under alkaline conditions, although the extinction coefficients are quite

different. Landsteiner & Chase [1936] have prepared from pepsin, by a process involving heating, a polysaccharide reacting with blood group A immune sera and this polysaccharide had rather different properties. The amount of polysaccharide contributed by the enzyme in the protein digests was negligibly small.

Initial effects of enzymes on proteins

A preparation of seroglycoid containing 6.3 % of carbohydrate was subjected to the action of pepsin at pH 1.6 for only 5 min. at 45°. The mixture was then neutralized and fractionated with $(\text{NH}_4)_2\text{SO}_4$. The properties of the fraction are summarized in Table VI.

Table VI. *Properties of fractions of seroglycoid digested for 5 min.*

Saturation with $(\text{NH}_4)_2\text{SO}_4$ %	Amount of fraction g.	Carbohydrate content %	Optical rotation $[\alpha]_{5461}$	Heat coagulability
33	0.28	3.0	- 90°	+
50	0.88	6.6	- 90°	+
66	0.26	15.0	- 94°	-
100	0.18	24.0	- 66°	-

It will be seen that this very brief treatment with pepsin had split seroglycoid into fractions of entirely different properties from the original protein. The last fraction in the table is not coagulated on heating and has a very high carbohydrate content, properties which render it of considerable interest which will be discussed later.

The behaviour of globoglycoid after brief pepsin digestion was different. After 5 min. treatment with pepsin at 43° about half the protein was precipitated by one-third saturation with $(\text{NH}_4)_2\text{SO}_4$ although the original material required 45-55 % saturation for precipitation, but it was not found possible to isolate a high-carbohydrate fraction as in the case of seroglycoid.

Antitoxic sera have been purified by peptic digestion [Parfentjev, 1936; Pope, 1939]. A pseudoglobulin solution containing haemolytic streptococcus antitoxin was submitted to a process similar to that of Pope [1939]. After brief peptic digestion at room temperature in the presence of tricesol some inactive proteins were removed by heat coagulation at 55° and the antitoxin was precipitated by 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$. Addition to the mother liquors of more $(\text{NH}_4)_2\text{SO}_4$ precipitated a sticky gum which dissolved readily when dialysed. This material comprised some 5 % of the original protein, it gave a strongly positive Molisch reaction and contained over 8 % carbohydrate whilst the original protein contained only half this amount. The material was not precipitated by 3 % trichloroacetic acid and the main bulk remained uncoagulated after heating, but it was precipitated by phosphotungstic acid.

DISCUSSION

That the polysaccharide present in proteins forms an integral part of the protein molecule and is not merely a loosely bound contaminant is shown by the difficulty of removing this "bound carbohydrate". Prolonged fractionation with salts, isoelectric precipitation, dialysis, heat coagulation and even specific flocculation between antigen and antibody all failed to remove the carbohydrate present in serum proteins [Hewitt, 1934] and Neuberger [1938] has shown that recrystallization and ultra-filtration failed to remove the carbohydrate from egg-albumin. In order to separate the carbohydrate moiety from

the protein drastic chemical hydrolysis or proteolytic enzyme digestion is necessary. It is not proposed to review historically the development of knowledge of the nature of the carbohydrate group present in proteins but it should be mentioned that the presence of glucosamine was detected very early [Seeman, 1898; Langstein, 1902] and later mannose was found [Fränkel & Jellinek, 1927; Levene & Mori, 1929; Rimington, 1929; 1931; Neuberger, 1938]. Modification of the Tillmans & Philippi [1929] orcinol colour reaction led Sørensen & Haugegaard [1933] to conclude that the carbohydrate present in serum proteins is galactose-mannose-glucosamine. The high figures of Sørensen [1938] herself for glucosamine are not in agreement with this view, but it is possible that all her glucosamine figures are too high since her ratio of glucosamine to mannose in ovalbumin is 0.82 compared with Neuberger's [1938] ratio of 0.5.

Neuberger [1938] has evolved a method of isolating the polysaccharide from protein digests in extremely good yield and of a high degree of chemical purity. The object of the present method was to prepare a product which should have suffered the very minimum of chemical change so that the final product should approximate to the complex present in the protein molecule even at the expense of chemical purity and, to this end, even the mild acetylation and deacetylation used by Neuberger has been omitted. It is hoped that step-wise enzymic digestion followed by extremely mild and simple methods of isolating the product may eventually lead to further knowledge of the mode of linking of polysaccharide in the protein molecule.

In the present work hexosamine and acetyl radicle determinations are in agreement with the presence in serum proteins of galactose-mannose-acetyl hexosamine. The colour reactions with orcinol and sulphuric acid of both the intact proteins and of the separated polysaccharides also agree with the presence of that carbohydrate unit. Colorimetric evidence can never be considered as entirely satisfactory, but in view of the mixture of sugars present it seems that the complete elucidation of the structure of serum protein polysaccharides is likely to be a matter of some complexity requiring considerable amounts of material.

The presence of acetylhexosamine in the polysaccharide is not surprising since it is a constituent of the polysaccharides obtained from a number of natural sources. Like the polysaccharides obtained from *B. dysenteriae* [Morgan, 1937] and ovalbumin [Neuberger, 1938] by methods avoiding the use of drastic chemical hydrolysis, the carbohydrate from serum proteins still contains some non-carbohydrate nitrogenous material. That it is not due to contamination with undigested protein is shown by the absence of various colour reactions and by the ultra-violet absorption spectra which differs from those of serum proteins in the absence of bands corresponding to tyrosine and tryptophan. Rimington [1931] experienced great difficulty in removing nitrogenous material present in the polysaccharide complex even when he used drastic hydrolytic processes which also decomposed some of the carbohydrate itself.

Seroglycoid and serum mucoid

The behaviour of serum proteins when subjected for a very brief period (e.g. 5 min.) to the action of pepsin is of some interest. In the case of seroglycoid, the protein was split into fractions of entirely different properties. One of these fractions was precipitable between the limits of 66 and 100% saturation with $(\text{NH}_4)_2\text{SO}_4$, it was not heat-coagulable and it contained some 24% of carbohydrate. It is remarkable that these properties correspond to those ascribed to serum mucoid [Zanetti, 1897; Bywaters, 1909; Rimington, 1933]. Despite

several attempts the present author has failed to detect the presence in normal blood serum of a protein corresponding to mucoid and it seems probable that the mucoid previously described was due to the decomposition of seroglycoid either by the drastic method of heating with acid or by the action of proteolytic enzymes present in serum. It has been shown above that after brief treatment with proteolytic enzymes material with properties corresponding to mucoid may be obtained from seroglycoid, and the protein-disaggregating effect of the fibrinolysin present in serum has been described by Pope [1939].

Globoglycoid did not yield a product of this kind when subjected to brief proteolysis and this presents additional evidence of the difference between seroglycoid and globoglycoid.

SUMMARY

1. A series of digestions with pepsin and trypsin followed by acid acetone precipitations provide a simple method of isolating the carbohydrate complex present in the protein molecule.

2. A study has been made of some of the properties of the polysaccharides present in the serum proteins seroglycoid and globoglycoid.

3. The evidence so far available is compatible with the view that the carbohydrate present in serum proteins contains galactose-mannose-acetylhexosamine.

4. Like polysaccharides from other natural sources the preparation contains non-carbohydrate nitrogenous material which may be removed by more drastic chemical procedures.

5. Very brief treatment of seroglycoid with pepsin yields a partial breakdown product with the same properties as those ascribed to "serum mucoid" and it is suggested that this latter product, which could not be detected in normal serum, is produced by disaggregation of seroglycoid.

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CLXXXV. ACETYLCHOLINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

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THE substance of this paper forms a continuation of our work [Mann *et al.* 1938, 1; 1939] on the conditions underlying acetylcholine formation in brain tissue *in vitro*. It demonstrates the influence of glucose at low concentrations, affords an explanation of results recently obtained on the acetylcholine contents of the brains of insulin-treated animals [MacIntosh, 1939] and indicates why Stedman & Stedman [1939] were unable to observe the accelerating effect of glucose on acetylcholine formation in brain.

Technique

The method of estimation of acetylcholine (free, "bound" and total), and the preparation and examination of brain tissue are described fully in previous papers [Mann *et al.* 1938, 1; 1939]. The reader is referred to these papers for full experimental details. All estimations are given in terms of acetylcholine chloride.

The effects of glucose at varying concentrations on acetylcholine synthesis by brain slices

It is now known [Quastel *et al.* 1936; Mann *et al.* 1938, 1; 1939] that in the presence of glucose and under aerobic conditions, brain tissue, whether sliced or minced, synthesizes acetylcholine. In the absence of glucose the synthesis is small or negligible. The importance of glucose for acetylcholine production has been confirmed by MacIntosh [1938] in perfusion experiments on intact ganglia. It is further known [Mann *et al.* 1939] that the addition of K ions (0.031 *M*) to a medium containing eserine, and in which intact brain slices are respiring, brings about a large increase in the rate of formation of acetylcholine. The total ester formed in 1 hr. at 37° may reach a value of over 40 μ g. (acetylcholine chloride) per g. wet weight of tissue.

Experiments have now been carried out on the effects of glucose at varying concentrations on the rate of acetylcholine formation by intact rat brain slices respiring in a medium containing eserine and 0.031 *M* KCl. The results are shown in Table I, where it will be seen that low concentrations of glucose are

Table I

Rat brain slices in bicarbonate-Locke-eserine medium, containing KCl to give final concentration of 0.031 *M*. 95 % O₂ + 5 % CO₂. 1 hr. 37°.

Exp. ...	Total acetylcholine formed, μ g./g.				
	A	B	C	D	E
Medium with no added glucose	5.1	5.9	5.3	3.5	7.8
+ 5 mg. glucose/100 ml.	12.8	—	—	—	11.0
+ 10 mg. glucose/100 ml.	14.5	—	—	—	17.0
+ 20 mg. glucose/100 ml.	26.7	33.3	33.3	27.9	—
+ 192 mg. glucose/100 ml.	—	33.7	—	38.3	—

(1506)

effective in securing acetylcholine synthesis. In the absence of added glucose, or in the presence of the very small quantities still remaining in the washed brain slices, there is but little formation of acetylcholine. The addition of glucose at a concentration of only 5 mg./100 ml. brings about a definitely increased rate of synthesis. A glucose concentration of 20 mg./100 ml. brings about a rate of acetylcholine formation which may be nearly maximal.

This remarkable activity of glucose in securing acetylcholine synthesis in the central nervous system probably accounts for the finding of MacIntosh [1939] that the brains of insulin-convulsed rats contain as much acetylcholine as those of normal animals. Convulsions of the animal may take place at blood sugar concentrations which are still capable of giving rise to acetylcholine formation. The experiment described in Table II confirms those of MacIntosh and also shows that the brains of insulin-convulsed animals have retained their ability to synthesize acetylcholine in presence of glucose.

Table II

A rat brain was minced and suspended in ten times its volume of eserized Locke solution. 1 ml. of the suspension was added to 2 ml. phosphate-eserine-Locke medium (with glucose added) and shaken in air at 37° for 1 hr., after which estimations of acetylcholine were made. The same procedure was carried out with the brain of an insulin-convulsed rat. Acetylcholine estimations were also carried out on the suspension before the incubation at 37° was commenced; these are the initial estimations.

	Total acetylcholine, $\mu\text{g./g.}$	
	Normal rat brain	Insulin- convulsed rat brain
Initial estimations	5.9	5.1
Medium + glucose (0.01 M)	21.4	18.8

*The effects of fructose, mannose and galactose on acetylcholine formation
by brain slices*

Of the three sugars, fructose, mannose and galactose, only mannose approaches glucose in its activity in bringing about acetylcholine synthesis. These sugars were examined at a concentration of 20 mg./100 ml. A low concentration was employed so as to afford comparison with glucose which is very active at this concentration and also to avoid complications due to the possible presence of traces of glucose as an impurity in the sugar (especially the fructose¹) preparations. Typical results are quoted in Table III. These results show a

Table III

Rat brain slices. Bicarbonate-eserine-Locke medium $\text{K}^+ = 0.031 \text{ M}$. 95% $\text{O}_2 + 5\% \text{ CO}_2$. 1 hr. 37°.

Medium	Total acetylcholine, $\mu\text{g./g.}$
	$\mu\text{g./g.}$
Medium	3.5
+ 20 mg. glucose/100 ml.	27.9
+ 20 mg. fructose/100 ml.	7.2
+ 20 mg. galactose/100 ml.	5.4
+ 20 mg. mannose/100 ml.	20.0

small effect of fructose in increasing acetylcholine formation, but we doubt whether this finding is a real one, for in one or two experiments we have failed to observe even this small effect. It is also possible that traces of glucose in the

The fructose was a B.D.H. (glucose-free) preparation.

fructose preparation might yield an irregular small accelerating effect. The small effects of fructose and of galactose, as compared with that of glucose in securing acetylcholine synthesis, confirm the observations of Kahlson & MacIntosh [1939] on the effects of sugars on acetylcholine production in perfused ganglia. According to these authors galactose has 5% of the activity of glucose whilst fructose is inert.

The failure of Stedman & Stedman to confirm the accelerating effect of glucose on acetylcholine synthesis by brain tissue

Stedman & Stedman [1939], in a recent paper, have failed to confirm the accelerating action of glucose on acetylcholine synthesis in isolated brain and state that their "results are in complete contradiction with those of Mann *et al.* [1938, 1]". Moreover they state that their "experiments were carried out under conditions as similar as possible to those of Mann *et al.*" It is necessary to consider their experimental method in some detail.

They ground 1 g. portions of minced rat brain tissue in 0.5 ml. saline- eserine solution, and washed these into flasks with 5 ml. phosphate-Ringer solution. To one flask was added 1 ml. saline and to the other was added 1 ml. saline containing 2 mg. glucose. The flasks, after filling with O_2 , were shaken at 37° for 2 hr. It was found that the yield of acetylcholine was identical in both cases—the yield being at least 18 $\mu\text{g./g.}$, this being expressed in terms of acetylcholine bromide.¹ This yield is 14.4 $\mu\text{g./g.}$ in terms of acetylcholine chloride.

It is easy to see that the failure of Stedman & Stedman to show an accelerating effect of glucose on acetylcholine production was due to their use of relatively large quantities of rat brain tissue. Such large quantities of tissues, which were not washed free of metabolites, introduced considerable concentrations of glucose and lactic acid into the phosphate-Ringer medium, the concentrations being sufficient to yield practically optimal synthesis under the experimental conditions employed. Moreover Stedman & Stedman made no observations of the rates of O_2 uptake by the suspensions in the two flasks. It is possible that under the conditions they employed the rates of O_2 uptake were so great as to be limited by the diffusion of O_2 into the medium and not by the concentration of metabolites present. Under such conditions the addition of a further quantity of metabolite (glucose) would not be expected to add materially to the effect of the glucose or other metabolites already present.

We have carried out experiments on the rates of formation of acetylcholine using varying quantities of minced brain tissue, in the presence and in the absence of added glucose. Typical results are noted in Table IV. It will be seen that the effect of added glucose is less when a large quantity of minced brain is taken than when a small quantity is employed. This would be expected in view of the quantity of glucose (or lactic acid) already present in the tissue. Moreover the yield of acetylcholine with small quantities of tissue in presence of glucose is higher than with large quantities of tissue in presence of glucose. This is probably due to the higher rates of O_2 consumption (per unit weight of tissue) which are secured when relatively small quantities of tissue are employed. The figures (obtained with small quantities of tissue) quoted in Table IV are similar to those quoted in our earlier papers. It will be noted that the yield of acetylcholine with small quantities of tissue in the absence of added glucose is relatively high. This is partly due to synthesis brought about by metabolites still

¹ Dr Stedman has kindly informed us that all his figures are expressed in terms of acetylcholine bromide.

Table IV

Exp. A. Two rat brains (3 g.) were chopped in presence of 0.6 ml. 1/100 eserine-sulphate-Locke solution. This was added to phosphate-Locke solution to give a total vol. of 9 ml. Appropriate volumes of the suspension were placed in Warburg flasks in which were placed solutions (saline, or phosphate-saline) which were either free from glucose or contained 0.01 *M* glucose. Total vol. in Warburg flasks = 3.2 ml. Air. 37°. 3 hr.

Medium	Amount of minced tissue present	Total acetylcholine $\mu\text{g./g.}$
Saline	1.0 g. + no glucose added	10.0
Saline	1.0 g. + glucose (0.01 <i>M</i>)	13.5
Phosphate-saline	0.1 g. + no glucose added	10.9
Phosphate-saline	0.1 g. + glucose (0.01 <i>M</i>)	16.5

Exp. B. Two rat brains were chopped and suspended in 2 vols. Locke medium. Appropriate quantities of the suspension were added to phosphate-eserine-saline media, with and without glucose, in Warburg flasks. Air. 37°. 2 hr.

Wt. of tissue taken g.	Total acetylcholine, $\mu\text{g./g.}$	
	No glucose added	Glucose (0.01 <i>M</i>) added
1.0	6.8	7.0
0.2	11.0	17.0
0.1	11.6	20.4

present and partly due to the acetylcholine present initially, or preformed, in the tissue before the commencement of the experiment.

We have carried out, also, experiments with relatively large quantities of tissue which have been washed with Locke solution. It is a difficult matter to remove metabolites from minced brain tissue by washing without removing at the same time factors necessary for the oxidation of glucose. Hence only a partial removal of metabolites is possible if the respiratory system is to be left intact. Results, in Table V, show that the glucose effect in increasing acetylcholine formation is greater with washed than with unwashed brain, and moreover that the acetylcholine yields by washed and unwashed brains, in presence of glucose, under the experimental conditions employed are the same.

Table V

Rat brains were minced and suspended in Locke solution. One part was washed with Locke solution by centrifuging. Appropriate volumes of unwashed and washed brain suspensions were placed in phosphate-saline medium with and without glucose. O₂. 37°. 2 hr.

	Total acetylcholine, $\mu\text{g./g.}$
0.8 g. unwashed brain. No glucose	7.5
0.8 g. unwashed brain + glucose (0.01 <i>M</i>)	12.5
0.8 g. washed brain. No glucose	4.5
0.8 g. washed brain + glucose (0.01 <i>M</i>)	12.5

Finally, using unwashed minced brain tissue, we have shown that the acetylcholine production is less in an atmosphere of N₂ than in one of O₂, whether glucose be added or not. Such a diminution in acetylcholine formation under anaerobic conditions when using relatively large quantities of unwashed brain tissue is to be expected, since we have shown in earlier work that the synthesis of acetylcholine due to the presence of glucose is suppressed in the absence of O₂. These results are shown in Table VI.

Table VI

Rat brains were minced and suspended in 2 vols. Locke solution. Appropriate quantities were placed in Warburg vessels containing phosphate-*eserine*-Locke medium with or without glucose. Two vessels were filled with O₂, and two with N₂. Incubation for 2 hr. at 37°.

		Total acetylcholine, μg./g.
0.8 g. brain tissue. No glucose	O ₂	9.0
0.8 g. brain tissue + glucose (0.01 M)	O ₂	15.0
0.8 g. brain tissue. No glucose	N ₂	3.0
0.8 g. brain tissue + glucose (0.01 M)	N ₂	4.4

The observations which have just been recorded make it clear that if a relatively large quantity of minced brain tissue is employed under our experimental conditions, a relatively high rate of acetylcholine formation may take place, the rate being dependent on the amount of glucose present initially in the tissue and on the conditions of oxygenation. The addition of glucose may, or may not, materially increase this rate.

Hence the observation of Stedman & Stedman that the addition of glucose did not increase the acetylcholine formation in isolated brain tissue is one which might have been expected considering the experimental conditions employed and is certainly not a "complete contradiction" of our results.

It is clear that Stedman & Stedman agree that acetylcholine is produced when minced rat brain, suspended in a phosphate-Ringer-*eserine* medium, is incubated in O₂ at 37°. Yet earlier on in their paper they say: "Apparently water, like certain organic solvents, destroys the mechanism responsible for the production of acetylcholine in brain tissue." It is difficult to reconcile a statement such as this with their own observations on acetylcholine formation in rat brain tissue suspended in Ringer solution.

Yield of acetylcholine

Stedman & Stedman [1939] give the yield of acetylcholine in their experiment with rat brain tissue suspended in Ringer solution as 14.4 μg./g. (acetylcholine chloride). This amount, as they say, is of the same order as the amounts obtained by ourselves when using minced brain tissue in a phosphate medium. Usually small quantities of tissue in presence of glucose give higher figures, whilst larger quantities of tissue in absence of glucose give smaller figures—but the order of magnitude is the same. The reasons for this have already been given.

Our method of analysis of total acetylcholine depends on acidification of the suspension of the brain tissue with subsequent neutralization, the method being relatively rapid and simple [for details see Mann *et al.* 1939]. Stedman & Stedman use a method which they admit to be long and tedious and which obviously gives results of the same order as our own. For this reason we have adhered to the method we have usually used and have not carried out routine analyses by the method of Stedman & Stedman. We have, however, carried out numerous control experiments to show that estimations of acetylcholine based on extraction of the tissue with 10% trichloroacetic acid do not give results markedly higher than those obtained by our method.

It is clear that the yield of acetylcholine obtained by minced rat brain tissue suspended in a phosphate-Ringer medium after incubation at 37° for 2 hr. is small compared with that obtained by rat brain slices incubated in a bicarbonate-glucose-Locke-0.03 M K⁺ medium at 37° for 1 hr. In the latter case yields varying from 30 to 50 μg./g. have been found [Mann *et al.* 1939; see also Table I].

The yields of acetylcholine from ether-soaked or chloroform-soaked brains as used by Stedman & Stedman are only a small fraction (one-third to one-quarter) of the yields obtained when rat brain slices are allowed to respire in a bicarbonate-glucose-potassium medium.

“Bound” acetylcholine

We [1938, 1] have shown that repeated washing of minced brain or of brain slices fails to extract all the acetylcholine and that a form of acetylcholine is left which is not attacked by choline esterase, but which is rapidly broken down at room temperature on treatment with acid (*pH* 2–3). This form of acetylcholine is referred to by us [1939] as “combined” or “bound” acetylcholine. The fact that such a combined ester, easily split by acid, exists in brain had been shown practically at the same time, and independently, by Loewi *et al.* [1938]. We [1938, 1; 1939] have already stated that this “bound” acetylcholine is probably identical with that described by Loewi and with that which Corteggiani [1937] has shown to give rise to free acetylcholine on heating at 70° for 3 min. We found [1938, 1] that “bound” acetylcholine is synthesized in brain tissue when the latter is allowed to respire in presence of glucose, lactate or pyruvate, in an eserine medium, but under anaerobic conditions or in the absence of a suitable metabolite no synthesis takes place. “Bound” acetylcholine is also synthesized by brain tissue under the proper physiological conditions in the *absence* of eserine, although no free acetylcholine can be detected. It was also shown [1938, 1; 1939] that the formation of free acetylcholine most probably takes place through the intermediate formation of “bound” acetylcholine which was therefore referred to as acetylcholine-precursor. The reader is referred to the papers in question for further details concerning the formation and properties of “bound” acetylcholine.

“Bound” acetylcholine is not only broken down to produce free acetylcholine after acid treatment but it is also broken down rapidly on shaking in aqueous media with chloroform, the amounts of free acetylcholine liberated being approximately the same. Details are given in our earlier paper [1938, 1]. It has been our custom when treating brain tissue suspension with chloroform (0.2 ml. chloroform to 5 ml. suspension) to leave the mixture at room temperature for 1 hr. But it may easily be shown that the “bound” acetylcholine is broken down in 10 min. at room temperature after the tissue is shaken with chloroform and that longer contact with chloroform leads to no appreciable increase in free acetylcholine. A typical result is shown in Table VII.

Table VII

Rat brain slices incubated aerobically in bicarbonate-Locke-glucose-eserine medium. 1 hr. 37°. Then washed in Locke solution to remove free acetylcholine. Divided into two equal lots, one shaken with chloroform and left at room temperature for 10 min. and the other shaken with chloroform and left at room temperature for 30 min. Chloroform removed *in vacuo* and the liberated free acetylcholine estimated.

	Acetylcholine liberated, $\mu\text{g.}/\text{g.}$
After shaking with chloroform and standing for 10 min. at room temperature	12.0
After shaking with chloroform and standing for 30 min. at room temperature	12.0

The same phenomenon occurs with the “bound” acetylcholine which is present in brain tissue freshly obtained from the animal. It can scarcely be

argued that the free acetylcholine rapidly formed after treatment with acid or with chloroform is due to synthesis brought about by these reagents under our experimental conditions. Hence the "bound" acetylcholine in fresh brain tissue, prior to incubation, is preformed and has been referred to by us as the preformed acetylcholine precursor. Stedman & Stedman [1939], however, dispute the existence of the preformed combined form of acetylcholine or of the preformed precursor. These authors seem to be confused over the definition of the preformed complex. They appear to think that we state that the increase of acetylcholine production obtained after acidification of a brain suspension which has been incubated in O_2 at 37° for some time is a demonstration of the existence of, and a measure of the amount of, the "preformed precursor" in the tissue after incubation. This, of course, is not true. The measure of preformed complex cannot be obtained *after* incubation of the tissue, only *before* incubation. The amount of complex found after incubation is referred to as the "bound" or combined acetylcholine formed, and usually experiments are carried out to ascertain the initial quantities of both "bound" and free acetylcholine. Moreover, Stedman & Stedman state that we "only measure the acetylcholine content of the suspension medium" but it must be obvious that measurements of total and "bound" acetylcholine are only made on the suspension medium when the tissue suspended in it has been treated with acid or chloroform etc. to accomplish the breakdown of the complex.

Apparently Stedman & Stedman [1939] admit the existence of Loewi's "bound" acetylcholine which is broken down by acids as well as by alcoholic HCl. They do not admit the existence of the preformed acetylcholine complex we have described because (a) we state that it is broken down by chloroform as well as by acid treatment and (b) they [1939] find that incubation of chloroform-treated brain leads to greater acetylcholine production than is found when brain tissue is treated at once with trichloroacetic acid or acid alcohol.

A feature, however, of the work of Stedman & Stedman is their use of large quantities of brain tissue in the absence of added aqueous media. In their experiments with chloroform-treated ox brain they use 10 g. brain tissue at a time, this amount being macerated with 5 ml. of a solution of eserine in chloroform. They find that an initial figure of $1.3\text{--}1.4\mu\text{g./g.}$ acetylcholine bromide is increased by incubation for 2 hr. at room temperature in presence of chloroform to $6\mu\text{g./g.}$ acetylcholine bromide. It is possible—and indeed probable as suggested by us earlier—that some synthesis, due to the glucose or lactic acid present in the brain tissue employed, is taking place at room temperature, in spite of the known deleterious action of chloroform on acetylcholine synthesis at 37° [Mann *et al.* 1938, 1].¹ Whether chloroform—or ether—under certain conditions can accelerate this synthesis, is a question which will be discussed shortly.

It is apparent from what has been said, that the increased formation of acetylcholine in chloroform-treated brain incubated at room temperature is not evidence against the existence of the preformed "bound" acetylcholine which we have described. The evidence submitted in our earlier work [1938, 1] and confirmed here shows that the complex in question exists, that "bound" acetylcholine is broken down on shaking with chloroform (see Table VII, as well as earlier work), and that it is probably identical with the complex described by other workers [Corteggiani, Loewi].

It should be emphasized that "bound" acetylcholine is synthesized by

¹ It is obvious, since the brain tissue is not diluted by the presence of aqueous media the active substrates (e.g. glucose or its equivalent of lactic acid) must still be present at approximately the same high concentrations as found in brain *in vivo*.

isolated brain examined under optimal physiological conditions in the absence of eserine. The free ester is not detectable owing to its destruction by the choline esterase present. Clearly such synthesis must be taking place *in vivo* and accounts for the fact that acetylcholine can be found in brain when taken freshly from the animal. This ester is mostly in the "bound" condition, for only traces of the free ester can exist in presence of the choline esterase of the tissue. The complex is the preformed precursor or preformed "bound" acetylcholine, whose existence Stedman & Stedman apparently dispute.

We showed [1938, 2] that acetylcholine formation by chloroform-treated ox brain is less at 37° than at 18° and this fact Stedman & Stedman [1939] have confirmed. They could not confirm our statement that the synthesis is no less at 0 than at 37°. Conceivably some difference in technique is responsible for the divergence of opinion here, but the significant point that the formation of acetylcholine in chloroform-treated brain is greater at room temperature than at 37° is conceded. Stedman & Stedman explain this by stating that chloroform has a dual action in brain tissue, a facilitating action on acetylcholine formation accompanied by a destruction of the mechanism responsible for its formation. We [1938, 1] had already pointed out that chloroform exerts a dual action, for it breaks down "bound" acetylcholine into the free ester and inhibits the synthesis of the ester at 37°.

The effects of ether

Stedman & Stedman [1939] turn from a consideration of chloroform to that of ether, "the effect of which" they say "must clearly be similar to that of chloroform". With ether-treated brain they find that the yield of acetylcholine is greater than when the tissue is treated with chloroform and moreover that the effect of ether at 37° is greater than at room temperature. They find as large effects with rat brain as with ox brain.

In repeating this work we have confined ourselves entirely to rat brain since this can be obtained quite fresh.

We have confirmed the fact that brain tissue macerated with half its volume of a solution of eserine in ether produces more acetylcholine than a similar brain ground with a solution of eserine in saline after incubation at 37° under aerobic conditions. Typical results are given in Table VIII. They are of the same order

Table VIII

Two rat brains were chopped and weighed out into 1 g. lots. One lot was ground with 0.5 ml. saline-eserine sulphate (1/800). Another lot was ground with 0.5 ml. ether-eserine base (1/1000). Both were placed in stoppered flasks and incubated in air for 2 hr. at 37°. Total acetylcholine was estimated as the chloride at the end of this period by acid treatment, ether being finally removed by evaporation at room temperature *in vacuo*.

	Total acetylcholine, $\mu\text{g./g.}$	
	Exp. A	Exp. B
Brain incubated with saline eserine	5.0	6.2
Brain incubated with ether eserine	8.0	10.0

of magnitude as those given by Stedman & Stedman who cite as their presumably highest yield with rat brain treated with ether 16 $\mu\text{g./g.}$ (as acetylcholine bromide, i.e. 12.8 $\mu\text{g./g.}$ acetylcholine chloride). It may be emphasized again that this value is small compared with the yields of acetylcholine produced by rat brain slices incubated in bicarbonate-glucose- K^+ (0.031 *M*) medium (see Table I).

The fact that the presence of ether in minced brain should increase the acetylcholine formation is of interest in view of the fact that the addition of ether

to washed minced whole brain suspended in a glucose-saline or Ringer medium and incubated at 37° aerobically prevents acetylcholine synthesis. This result is shown in Table IX. Evidently when ether is shaken with a brain suspension

Table IX

Three rat brains were chopped, weighed into four 0.5 g. lots each of which was transferred to a centrifuge tube and each washed by centrifuging with 5 ml. eserine Locke solution. The washed tissues were mixed with 0.2 ml. 1/400 eserine sulphate solution. To two of the tubes were added 0.25 ml. ether and the contents were transferred to Warburg flasks containing media mentioned below. Incubation for 1 hr. in air at 37°.

Medium	Total acetylcholine formed, $\mu\text{g./g.}$
Phosphate-Locke	5.0
Phosphate-Locke-glucose (0.01 M)	10.0
Phosphate-Locke-ether	4.0
Phosphate-Locke-ether-glucose (0.01 M)	4.0

in an eserine-phosphate medium, the ability of glucose to induce synthesis of acetylcholine is inhibited. Ether is therefore more toxic to the synthetic processes in brain when it is shaken in an aqueous medium, than when it is macerated with minced brain and this is incubated aerobically in the absence of an aqueous medium. The inhibitory effect of ether on the respiration of brain tissue incubated in a phosphate-glucose-Ringer medium has been shown by Jowett & Quastel [1937].

It is to be noted that the two methods of administering ether to brain tissue differ greatly. When brain tissue is ground in a mortar with half its volume of ether, most of the latter disappears by evaporation, and but a fraction is left adhering to the brain substance. It is questionable whether the ether has penetrated all cells of the tissue. When it is shaken in an aqueous suspension with ether as in the Warburg manometric technique the brain cells are constantly exposed to a solution saturated with ether. Possibly in this case the penetration of ether into the cells, and therefore its destructive effects, are more marked than in the previous method which is that adopted by Stedman & Stedman.

It may be shown that ether, like chloroform or acid, when shaken with a suspension of broken up washed brain slices releases free acetylcholine from the "bound" ester. The breakdown of the "complex" into free acetylcholine takes place in 5 min. at room temperature. This result is shown in Table X.

Table X

Rat brain slices incubated in bicarbonate-glucose-eserine-Locke medium. 1 hr. 37°. 95% O₂ + 5% CO₂. Slices then well washed with saline eserine to remove free acetylcholine. Slices broken up and treated with (a) HCl to pH 2-3, for 5 min., then neutralized and centrifugate examined for acetylcholine, (b) ether (0.5 ml.) for 5 min., centrifuged, ether removed from centrifugate by evaporation *in vacuo* at room temperature and centrifugate then examined for acetylcholine.

	Acetylcholine found, $\mu\text{g./g.}$
(a) Acid treatment (5 min.)	14.2
(b) Ether treatment (5 min.)	15.8

Since it is evident that ether, like chloroform, is inhibitory to the synthesis of acetylcholine in brain when this tissue is examined in bicarbonate- or phosphate-Locke media, we have confined our experiments to the technique adopted by Stedman & Stedman to study the mechanism whereby ether accelerates acetylcholine synthesis in minced brain.

Action of ether on formation of free and "bound" acetylcholine

The accelerating action of ether on acetylcholine formation in minced brain incubated in air at 37° in the absence of the addition of an aqueous medium appears to lie entirely with the free acetylcholine, the "bound" ester being diminished in quantity. This is shown by the results given in Table XI. Thus,

Table XI

Three rat brains were minced and weighed out into portions of 0.8 g. tissue. One portion was ground in a mortar with 0.4 ml. saline-eserine and the other ground in a mortar with 0.4 ml. eserine ether. The two portions were placed in stoppered flasks and incubated aerobically at 37° for 2 hr. Then they were washed twice with saline-eserine, the centrifugates being examined for free acetylcholine. The washed residues were treated with acid to break down "bound" acetylcholine into the free ester which was estimated in the final centrifugate.

Acetylcholine found, $\mu\text{g./g.}$

	Free	"Bound"	Total
Brain treated with saline eserine	4.2	1.5	5.7
Brain treated with ether eserine	7.4	0.5	7.9

although the total acetylcholine is increased by the presence of the ether, it is the free acetylcholine in the tissue whose amount is actually increased. This conclusion is confirmed by the following experiment. Minced rat brain tissue was incubated aerobically at 37°, after maceration with saline and with ether, no eserine being added until the *end* of the incubation time. After this late addition of eserine it was found that the total acetylcholine was definitely *less* in the ether-treated brain than in the saline-treated tissue. This result is shown in Table XII,

Table XII

Four rat brains were minced and weighed out into portions of 0.8 g. tissue. One portion was ground with 0.4 ml. saline, another with 0.4 ml. ether, a third with saline-eserine and a fourth with ether-eserine. These were transferred to Warburg flasks, in two of which 0.2 ml. eserine saline were present in the side tube. Incubated at 37° for 2 hr. in O_2 . Eserine tipped in at the end of 2 hr.

		Total acetylcholine, $\mu\text{g./g.}$
Brain + saline	Eserine added after 2 hr.	3.0
Brain + ether	Eserine added after 2 hr.	1.0
Brain + saline	Eserine added initially	6.2
Brain + ether	Eserine added initially	11.7

where results when eserine was added initially are given for comparison. This finding, which apparently contradicts the conclusion that ether brings about an acceleration of acetylcholine formation, is easily understood when it is realized that the effect of ether is to increase the rate of formation of free acetylcholine. In the absence of eserine the latter is destroyed and only the bound acetylcholine is estimated when eserine is added at the termination of the experiment.

These effects of ether in presence and absence of eserine are remarkably similar to those of K ions noted in our last paper [1939], but they are on a much smaller scale.

We then decided to investigate the action of Ca ions on the ether acceleration of acetylcholine synthesis since we know [1939] that Ca ions neutralize the effects of K ions on the synthesis of the ester.

Action of Ca^{++} on acetylcholine formation in ether-treated brain

The addition of 0.02 M CaCl_2 to rat brain macerated with half its volume of ether eserine has the effect of greatly diminishing the accelerating action of ether on acetylcholine formation. Typical results are shown in Table XIII. Such a

Table XIII

Rat brains were minced and weighed out into lots of 0.8 g. tissue. Portions were ground in a mortar with (1) 0.4 ml. saline-eserine + 0.4 ml. saline, (2) 0.4 ml. saline-eserine + 0.4 ml. 0.08 M CaCl_2 , (3) 0.4 ml. ether-eserine + 0.4 ml. saline, (4) 0.4 ml. ether-eserine + 0.4 ml. 0.08 M CaCl_2 . These were transferred to stoppered flasks and incubated aerobically at 37° for 2 hr.

	Total acetylcholine formed, $\mu\text{g./g.}$		
	Exp. A	Exp. B	Exp. C
Brain + saline eserine	5.9	3.5	5.7
Brain + saline eserine + 0.02 M CaCl_2	5.9	—	—
Brain + ether eserine	7.1	6.7	7.9
Brain + ether eserine + 0.02 M CaCl_2	3.9	1.4	5.2*

* In this experiment the concentration of CaCl_2 was 0.01 M .

large diminishing effect on acetylcholine formation does not take place in the absence of ether. The further resemblance of the effect of ether to that which occurs with K ions is again obvious.

It must be noted, however, that the action of K ions on minced brain is much less pronounced than on brain slices [Mann *et al.* 1939] and it is not to be supposed on present evidence that K ions and ether are interchangeable in their effects on acetylcholine formation in minced brain.

The results suggest, however, that ether is acting on minced brain (in the absence of an aqueous medium) in a manner analogous to the action of potassium on brain slices. It was concluded by us [1939] that the latter depended on a change of cell permeability induced by the increased concentration of K^+ in the medium bathing the slices. In like manner we may imagine that ether exercises its effect by causing a changed permeability of the cells with which it comes into contact.¹ If this view is true then we may expect that the ether effect, like that of potassium, will only take place in the presence of a "synthesizing substrate" such as glucose. Unfortunately this cannot be tested by the technique adopted by Stedman & Stedman since it is difficult to wash away metabolites satisfactorily from large quantities of minced brain tissue without affecting the respiratory mechanisms of the tissue. Moreover such washing introduces aqueous media into the tissue which renders it more susceptible to the toxic action of the ether. The problem can, however, be attacked from a different point of view. Since it is known that acetylcholine synthesis due to the presence of glucose depends on aerobic conditions, it follows that if the view we have suggested is correct then the accelerating effect of ether will be considerably lower in N_2 than in O_2 .

Effects of anaerobic conditions on the ether acceleration of acetylcholine formation

The effect of substituting N_2 for O_2 in the atmosphere surrounding the ether treated brain greatly reduces the acetylcholine formation. Typical results are

¹ Similarly the relatively small enhancement of the rate of acetylcholine formation found with chloroform-treated brain when incubated, in the absence of aqueous media, at room temperature may be attributed to changes of cell permeability effected by the chloroform. Much of the argument applying to ether probably applies also to chloroform; the latter, however, is much more damaging than ether towards the synthesizing enzyme.

shown in Table XIV. Probably the yields found in N_2 would be still lower if all the O_2 still remaining in the tissue could be removed. It is experimentally difficult, however, to accomplish this.

Table XIV

Rat brains were minced and weighed out into lots of 0.5 g. Portions were ground with 0.25 ml. ether eserine, transferred to Warburg flasks one of which was filled with N_2 and the other with O_2 . Incubated for 2 hr. at 37° .

			Total acetylcholine formed, $\mu\text{g./g.}$	
Brain.	Ether eserine.	O_2	Exp. A	Exp. B
Brain.	Ether eserine.	N_2	11.7	7.5
			6.0	4.5

The observations recorded above indicate that the synthesis of acetylcholine in ether-treated minced brain depends on the presence of a metabolite such as glucose which is known to require aerobic conditions for synthesis of the ester. The action of ether in accelerating acetylcholine formation seems to us to be most easily explained on the same lines by which the K effect was interpreted, i.e. by a change in permeability of the cell. The reader is referred to our recent paper [1939] for full details of this.

Stedman & Stedman [1939] give no explanation of the ether effect beyond stating that one of the functions of the solvent is to act as vehicle for conveying the eserine into the tissue.

Acetoacetate as a possible precursor of acetylcholine in brain tissue

We have already shown [1938, 1, 2] that acetoacetate added to brain tissue respiring in a phosphate-Locke-eserine medium in O_2 at 37° does not yield acetylcholine at a rate which even approaches that brought about by the presence of glucose. Consequently we are loath to believe that acetoacetate is a precursor of acetylcholine¹. Stedman & Stedman [1939], however, find using their ether-treated minced brain technique that a yield of $17 \mu\text{g./g.}$ (acetylcholine bromide) is increased to $23 \mu\text{g./g.}$ by the addition to the tissue of 20 mg. sodium acetoacetate. In another experiment with ox brain a value of $8.7 \mu\text{g./g.}$ acetylcholine bromide was increased to $14.5 \mu\text{g./g.}$ by the addition of the same quantity of acetoacetate. They consider these results to be substantial evidence that acetoacetic acid is in fact a precursor of acetylcholine but they state they have failed to show a synthesis of acetylcholine from acetoacetate in aqueous media. Our own position is that we must regard accelerations of syntheses which take place only in ether- or chloroform-treated brains and which do not seem to occur in brain tissue bathed in a Ringer medium, as of questionable physiological significance so far as the mechanism of the biological formation of acetylcholine is concerned. It has not been proved, because acetoacetate enhances the effect of chloroform and of ether in increasing the rate of formation of acetylcholine in brain, that acetoacetate itself is converted into acetylcholine. Many factors appear to influence the action of chloroform and of ether on brain as the observations given above have shown and it may well be that acetoacetate is reacting in a physicochemical, rather than in a strictly chemical, sense. It is clear that further information is required before the conclusion can be accepted that acetoacetate is a direct precursor of acetylcholine.

¹ See also Kahlson & MacIntosh [1939] who have found acetoacetate to be ineffective as a promoter of acetylcholine synthesis in perfused ganglia.

SUMMARY

1. Glucose at low concentrations, e.g. below 20 mg. per 100 g. will bring about the synthesis of acetylcholine in intact rat brain slices.
2. Fructose and galactose have relatively small effects (that of fructose being doubtful) in securing acetylcholine synthesis, but mannose has an activity approaching that of glucose.
3. The failure of Stedman & Stedman to confirm the effect of glucose in enhancing acetylcholine production by brain is shown to be due to the limitations of their experimental technique.
4. The rate of formation of acetylcholine in brain tissue using the technique of Stedman & Stedman is much less than that obtained when intact rat brain slices respire in a bicarbonate-glucose medium containing $0.03\text{ }M\text{ }K^+$.
5. Preformed "bound" acetylcholine exists in brain tissue freshly obtained from the animal and the suggestion of Stedman & Stedman that it does not exist is based upon insufficient experimental evidence.
6. The effect of ether in enhancing acetylcholine formation in minced brain tissue when incubated at 37° in the absence of an aqueous medium is confirmed.
7. Ether is highly inhibitory to acetylcholine formation in brain tissue examined in a phosphate- or bicarbonate-Locke medium. It is less toxic when small quantities are macerated with brain in the absence of an aqueous medium.
8. In brain suspensions in aqueous media both ether and chloroform break down "bound" into free acetylcholine, the reaction taking place quickly (5 min.) at room temperature.
9. The accelerating action of ether on acetylcholine formation in minced brain, in the absence of an aqueous medium, lies entirely with the production of free acetylcholine, the amount of "bound" ester formed being diminished in quantity. The accelerating action of ether is diminished or removed by the addition to the tissue of $0.02\text{ }M\text{ }Ca^{++}$. The accelerating action is also greatly diminished by carrying out the experiment under anaerobic conditions. These effects closely resemble, though on a smaller scale, the action of K^+ on acetylcholine formation in brain slices. It is suggested that the explanation of the ether effect is similar to that advanced by us [Mann *et al.* 1939] for the accelerating action of K ions. The fact that the presence of O_2 greatly increases the formation of acetylcholine in ether-treated brain suggests that the same metabolites are responsible for the synthesis as those in brain examined under more physiological conditions, e.g. glucose or lactic acid.

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CLXXXVI. STUDIES IN DETOXICATION

III. THE USE OF THE GLUCURONIC ACID DETOXICATION MECHANISM OF THE RABBIT FOR THE RESOLUTION OF *dl*-MENTHOL

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RESOLUTION of racemic substances as a result of selective assimilation by living organisms is originally due to Pasteur [1858, 1860] who showed that *Penicillium glaucum* destroyed the *d*-tartrate in a solution of ammonium racemate leaving the *l*-isomeride. Examples of the action of micro-organisms in this manner are numerous [see Cohen, 1924].

The mammalian organism also deals with optical antipodes selectively and this fact has been utilized in a few cases to produce a partial separation of the antipodes, when they are excreted in the urine following their introduction into the body. Thus, when sodium *dl*-phenyl- γ -hydroxybutyrate is given to dogs, more *l*-isomeride than *d*- is found in the urine [Thierfelder & Schempp, 1917]; sodium *dl*-malate injected subcutaneously into rabbits gives *d*-malate in the urine [Tomita, 1921] and intravenous injection of sodium *r*-mandelate into cats results in the preferential excretion of the *l*-form at first, the *d*-form predominating later [Garry & Smith, 1938]. In the case of terpenes, it has been shown that the *dl*-alcohols of this series are detoxicated in the animal by conjugation with glucuronic acid and the glucuronide so formed contains more of one isomeride than of its enantiomorph [cf. Williams, 1938]. The object of the present investigation is to show that advantage can be taken of this asymmetric conjugation for the resolution of *dl*-menthol and possibly other terpenes.

The resolution of dl-menthol

Within recent years, *dl*-menthol has been resolved very successfully by Read and his co-workers [Read & Grubb, 1931; 1932; Clark & Read, 1934], whose methods depend upon fractional crystallization of the esters of *dl*-menthol with optically active acids. By Read's methods, *d*-menthol, previously regarded as a chemical curiosity, was easily obtained in excellent yields, together with *l*-menthol, from synthetic *dl*-menthol. More recently Barrow & Atkinson [1939] have resolved *dl*-menthol through its ester with (+)-tartranilic acid, although only *l*-menthol is obtained by this method. In the present paper the resolution of *dl*-menthol has been accomplished by a partially biochemical method which depends on glucuronide formation in the rabbit. Neuberg *et al.* [1928-9] have shown that *dl*-menthol can be resolved to give optically pure *l*-menthol (but not *d*-) through the glucoside; if *dl*-menthol is condensed with acetobromoglucose in the presence of quinoline, *l*-menthol tends to form an α -, whilst *d*-menthol tends

to form a β -glucoside, and these can be separated. If, however, *dl*-menthol is condensed in the presence of silver carbonate only β -glucosides are obtained and little separation of these is possible. Neuberg *et al.* also showed that emulsin, acting on *dl*-menthyl- β -glucoside, hydrolyses the *d*-form quicker than the *l*-, but optically pure menthols could not be obtained in this manner. In the present work the *d*- and *l*-menthylglucuronides obtained from the rabbit are both β -glycosides and can be separated by fractional crystallization. Three different procedures, described below, were adopted. Two of these gave *d*-menthol in about 10% yield (calc. on *dl*-menthol fed); the third method gave no further separation than that already performed by the organism of the rabbit.

(a) Oral administration of *dl*-menthol to rabbits results in the excretion of a diastereoisomeric pair, namely *d*- and *l*-menthyl- β -*d*-glucuronide, the former being more abundant than the latter. These diastereoisomerides as ammonium salts showed sufficient difference in solubility to be separated by fractional crystallization from water and ammonium sulphate solutions. The free acids could not be separated with any success. In this manner, 10 g. of the mixed ammonium menthylglucuronates could be made to yield 1-1.5 g. of ammonium *d*-menthylglucuronate.

(b) When *dl*-menthol is fed to rabbits, the *l*-isomeride is oxidized to a greater extent than the *d*- [Williams, 1938]. It should, therefore, be possible to destroy most of the *l*-form by taking advantage of this selective oxidation. *dl*-Menthol was fed to rabbits and the excreted glucuronide (NH_4 salt had $[\alpha]_D$ ca. -28° , and contained 30-35% *l*-) was hydrolysed with dilute acid, the menthol formed being simultaneously steam-distilled. The main bulk of the menthol recovered had $[\alpha]_D$ ca. $+20^\circ$ in alcohol. During this hydrolysis it was observed that the earlier fractions of menthol had a higher dextrorotation than later ones (see Table III) indicating that the rate of hydrolysis of *d*-menthylglucuronide is greater than that of the *l*-form. The recovered menthol was now re-fed to rabbits and the excreted glucuronide ($[\alpha]_D$ ca. -10°) now contained 85-90% of the *d*-isomeride. One recrystallization of the ammonium salt of this glucuronide from water containing 5% ammonium sulphate gave mainly *d*-isomeride, 10 g. of the mixed salts yielding 5-7 g. of the *d*-salt.

(c) It was thought possible that if the urine excreted by rabbits fed with *dl*-menthol were collected periodically it might be found that preferential excretion of one of the diastereoisomerides took place. This, however, was not realized since all fractions obtained had the same rotation and contained the same proportion of *d*- to *l*-menthol (see Table II).

Optically pure *l*-menthylglucuronide was not obtained from the mixed glucuronide since the amount of it occurring in the latter is much smaller than that of the *d*-. In one experiment by the first method above, an ammonium salt showing $[\alpha]_D -84^\circ$ (i.e. 80% *l*-) was obtained.¹

It is interesting to note that all the known natural conjugated glucuronic acids are laevorotatory. *d*-Menthylglucuronide appears to be the first to be discovered with a dextrorotation, albeit a small one. There is no reason to believe that it has other than a β -glycosidic structure. Table I summarizes the rotations and melting points of the known glucuronides of *p*-menthan-3-ol.

¹ 12 g. of ammonium *l*-menthylglucuronate were isolated from rabbit urine after feeding 18 g. *l*-menthol. The purified salt (needles of monohydrate) showed $[\alpha]_D^{20} -103.4^\circ$ ($c=1.8$ in water). *l*-Menthylglucuronide forms crisp plates (monohydrate) with m.p. 94° and $[\alpha]_D^{20} -110.7^\circ$ ($c=2$ in alcohol).

Table I. *The glucuronides of p-menthan-3-ol*

Glucuronide	M.P.	$[\alpha]_D$ in alcohol	Author
<i>l</i> - α -Menthyl (synthetic)	130°	+ 52°	Bergmann & Wolf [1923]
<i>l</i> - β -Menthyl	94°	- 110°	See footnote on p. 1520
<i>d</i> - β -Menthyl	120°	+ 6°	This paper
<i>d</i> - β -isoMenthyl	126°	- 43°	Williams [1938]

Isolation of d-menthol

This menthol was obtained by acid hydrolysis of *d*-menthylglucuronide. The yield was almost quantitative and it had M.P. 41° and $[\alpha]_D + 49.4^\circ$ in alcohol (Read & Grubb [1934], give M.P. 42–43° and $[\alpha]_D + 49.6^\circ$). Pure *d*-menthylglucuronide was obtained by acidification of solutions of the ammonium salt obtained from the resolutions. If *d*-menthol is prepared by hydrolysis of this ammonium salt, a specimen which is not optically pure ($[\alpha]_D + 46^\circ$) is obtained. This ammonium salt was found to contain a small amount of an unknown menthanolglucuronide, which was easily separated during the conversion of the NH_4 salt into the free acid (see experimental). This unknown glucuronide crystallizes in needles and its NH_4 salt forms plates. This crystal habit is typical of *d*-isomenthylglucuronide and its NH_4 salt [Williams, 1938] and of ammonium *dl*-isomenthylglucuronide. In the case of the *d*- and *l*-menthylglucuronides, the free acids form plates and the NH_4 salts needles. This unknown glucuronide may be an *isomenthyl* derivative, since Read & Grubb [1932] state that commercial *dl*-menthol often contains an appreciable amount of *dl*-isomenthol.

EXPERIMENTAL

Isolation of ammonium menthylglucuronate. Rabbits (2–2.5 kg.) on a diet of 100 g. cabbage and 50 g. bran a day were each given 3 g. *dl*-menthol ($[\alpha]_D 0^\circ$ in alcohol) by stomach tube in about 15 ml. water warm enough to melt the menthol (M.P. 34–37°). The urine was collected for about 30 hr. and after filtration through muslin, was made slightly alkaline with a few drops of ammonia (sp.gr. 0.880), boiled and filtered to remove precipitated phosphates. The filtrate was treated with 50 g. of ammonium sulphate per 100 ml. and brought to the boil with occasional shaking. The boiling urine was then filtered hot through a coarse fast filter paper to remove a precipitate of protein which appeared to be of a mucoid nature. The filtrate was now kept overnight in the refrigerator and ammonium menthylglucuronate crystallized in needles which filled the solution. The yield of crude dry salt was 1.2–1.4 g. per g. *dl*-menthol fed and a typical preparation had $[\alpha]_D - 28.3^\circ$ ($c = 1$ in water).

The rate of excretion of menthylglucuronide. Rabbits were given 3 g. each of *dl*-menthol and the urine was collected periodically during 48 hr. The NH_4 salt was isolated from each fraction by the method indicated above. In order to ensure as complete a separation of the salt as possible, the time for crystallization was prolonged to 2 days at 0°. A control experiment to check the yield was performed, the urine being collected and worked up in one batch. The result of such an experiment using 6 rabbits is given in Table II. It can be seen from the table that the excretion of conjugated menthol is complete in about 30 hr. The bulk (75%) of the material is excreted in the first 12 hr.

From the rotations of the various fractions (Table II) it can be seen that the proportion of *d*- to *l*-menthylglucuronide is constant and there is no evidence of preferential excretion of either one of them. Little significance can be attached

Table II. *The rate of excretion of menthylglucuronide after dl-menthol in rabbits*

A. 6 rabbits given 3 g. *dl*-menthol each and the urine collected periodically.
 B. 6 rabbits given 3 g. *dl*-menthol each and the urine collected in one batch.

Time after feeding hr.	A		B	
	Yield dry NH ₄ salt g.	[α] _D in water	Yield dry NH ₄ salt g.	[α] _D in water
6	6.5	-26.6°	—	—
12	12.4	-25.8°	—	—
24	3.6	-27.1°	—	—
31	2.0	-16.5°	—	—
42	—	—	25	-28°
48	0.0	—	—	—
	Total 24.5			

to the low rotation of the last fraction since it is easy to lose a small amount of the more soluble *l*-menthylglucuronate. Owing to the high negative rotation of the latter ($[\alpha]_D - 103^\circ$), loss of a small amount will have a considerable effect on the rotation of the mixed salts.

Fractionation of the salt $[\alpha]_D - 28^\circ$. A typical resolution of this salt was carried out as follows: 10 g. of the salt were dissolved in 50 ml. water with warming; after an hour, 2.1 g. of a salt of rotation -16.6° were isolated. This material was now dissolved in 15 ml. of warm water, and on standing a small amount of material with $[\alpha]_D - 33^\circ$ separated. The latter was removed and a little solid ammonium sulphate was stirred into the solution; 0.7 g. of ammonium *d*-menthylglucuronate ($[\alpha]_D + 5^\circ$) separated. The filtrate from the fraction with $[\alpha]_D - 16.6^\circ$ yielded another 0.7 g. of the *d*-salt on further fractionation. Recrystallization from water of the salt with $[\alpha]_D + 5^\circ$ failed to alter its rotation. The more soluble fractions gave, on fractional precipitation with increasing concentrations of ammonium sulphate, a small amount of material with $[\alpha]_D - 84^\circ$ which, therefore, contained over 80 % *l*-isomeride (the rotations are in water).

Isolation of ammonium d-menthylglucuronate after feeding recovered menthol

(a) *Hydrolysis of the salt* $[\alpha]_D - 28^\circ$. 10 g. of the salt obtained after feeding *dl*-menthol were dissolved in 100 ml. water and 15 ml. of 2 *N* H₂SO₄ were added. The free menthylglucuronide was extracted from the aqueous solution with ether. The ether was removed and the residue taken up in 100 ml. of 0.4 *N* H₂SO₄. The menthylglucuronic acid was hydrolysed by a steam-distillation of 2 hr. duration, the menthol formed being simultaneously collected in the distillate. The residual solution was usually worked up for the preparation of glucurone. In one experiment in which 92 g. of the mixed salt were hydrolysed, the menthol

Table III. *Recovery of menthol after hydrolysis of ammonium menthylglucuronate* $[\alpha]_D - 28^\circ$

Fraction	Yield g.	[α] _D in alcohol
1	1.6	+28°
2	1.7	+24°
3	25.0	+20°
4	3.0	-3°

was collected in four fractions. The rotations of these fractions are given in Table III.¹ The significance of these figures has already been pointed out.

(b) *Isolation of the glucuronide after feeding recovered menthol.* 30 g. of the recovered menthol (fractions of low rotation were not used) were fed to 10 rabbits each receiving 3 g. of the crude material. After 2 days the urine was worked up in the usual manner and 34 g. of crude ammonium salt were isolated with $[\alpha]_D - 9.7^\circ$ in water.

The fractionation of this salt was carried out as follows: the salt (17.6 g.) was dissolved in 180 ml. water with heating and the solution filtered. Ammonium sulphate (10 g.) was stirred into the solution and 12 g. of crude ammonium *d*-menthylglucuronate ($[\alpha]_D$ 0 to $+1^\circ$ in water) were isolated. Treatment of the filtrate with 20 g. ammonium sulphate gave a second fraction (4.1 g.) with $[\alpha]_D - 36^\circ$ in water. Recrystallization of the second fraction from water yielded a small amount of an ammonium salt which crystallized in plates and had $[\alpha]_D - 53.3^\circ$ in water. The latter is probably ammonium *dl*-isomenthylglucuronate, since the authentic salt has been prepared by the author during another investigation (unpublished) and found to have $[\alpha]_D$ ca. -54° .

Preparation of d-menthol-β-d-glucuronide. Ammonium *d*-menthylglucuronate (5 g.) as obtained above was dissolved in hot water (100 ml.) and the solution filtered. The filtrate was treated with twice the calculated amount of 2*N* H₂SO₄. On cooling the solution to 0°, almost pure *d*-menthylglucuronide separated in large lustrous plates (yield 80%). After two recrystallizations from water and drying over CaCl₂, it melted at 120–122° (uncorr.) and had $[\alpha]_D^{20} + 6.4^\circ$ (*c* = 2 in abs. alcohol). It is easily soluble in alcohol, ether and hot water, sparingly soluble in cold water and dilute mineral acids and almost insoluble in light petroleum. It possesses one molecule of water of crystallization after drying at room temperatures over CaCl₂. (Found: C, 54.6; H, 8.35; H₂O, 5.3%; equiv. by titration, 350.4. C₁₆H₂₆O₇, H₂O requires C, 54.8; H, 8.6; H₂O, 5.1%; equiv. 350.2.)

On keeping the filtrate after separating *d*-menthylglucuronide as above for several days, a second crop of crystals was obtained. These consisted of minute needles and had $[\alpha]_D - 44^\circ$ in water (M.P. 107–109°). They contained glucuronic acid and on hydrolysis gave a terpene alcohol with a menthol-like odour. They have not been identified so far.

Ammonium-d-menthylglucuronate. A pure specimen of this salt was prepared by dissolving the pure *d*-acid in water and neutralizing with a few drops of ammonia (sp. gr. 0.880). On keeping the solution the salt separated as needles, melting with decomposition at 200–202° and soluble in warm alcohol ($[\alpha]_D^{20} + 8.1^\circ$, *c* = 1 in water). (Found: N, 3.8; H₂O, 5.2%. C₁₆H₂₇O₇NH₄, H₂O requires N, 3.8; H₂O, 4.9%.)

Preparation of d-menthol. Acid hydrolysis of the *d*-salt obtained directly from the resolutions does not give an optically pure menthol owing to the presence of an unknown terpene alcohol already referred to above. A specimen obtained in this manner had M.P. 34–36° and $[\alpha]_D^{20} + 46.4^\circ$ in alcohol. An optically pure *d*-menthol was obtained on hydrolysis of pure *d*-menthylglucuronide. The glucuronide (2 g.) was hydrolysed by steam distillation with 15 ml. 2*N* H₂SO₄. The menthol in the distillate was extracted with light petroleum, the solution filtered and evaporated. The *d*-menthol formed long needles (yield 0.85 g., almost quantitative) and had a fainter odour than its *l*-enantiomorph as has been observed by previous investigators. It had M.P. 41° (corr.) and

¹ The figures quoted in this table were obtained by Dr M. Stacey during the preparation of glucurone from menthylglucuronide prepared by the author.

$[\alpha]_D^{20} + 49.4^\circ$ ($c = 1.66$ in abs. alcohol; mean of three determinations). Read & Grubb [1934] give for the *d*- and *l*-menthols, m.p. $42-43^\circ$ and $[\alpha]_D \pm 49.6^\circ$ in alcohol.

Two new derivatives of *d*-menthol were prepared for comparison with the known *l*-derivatives. *d*-Menthylphenylurethane (*d*-menthyl carbanilate) was prepared by heating *d*-menthol with phenylisocyanate. It forms long silky needles from light petroleum, m.p. $112-113^\circ$ (corr.) and $[\alpha]_D^{20} + 75.7^\circ$ ($c = 1$, in CHCl_3). (Found: N, 5.3%. $\text{C}_{17}\text{H}_{25}\text{O}_2\text{N}$ requires N, 5.1%.) Kenyon & Pickard [1915] give $[\alpha]_D - 76.9^\circ$ (in CHCl_3) for the *l*-form. *d*-Menthyl-3:5-dinitrobenzoate was prepared from *d*-menthol and 3:5-dinitrobenzoyl chloride. It forms long fluffy needles from alcohol and has m.p. $153-154^\circ$ (corr.) and $[\alpha]_D^{20} + 77.1^\circ$ ($c = 0.8$, in CHCl_3). (Found: N, 7.7%. $\text{C}_{17}\text{H}_{22}\text{O}_6\text{N}_2$ requires N, 8.0%.) Read *et al.* [1933] give m.p. 153° and $[\alpha]_D - 79.4^\circ$ (in CHCl_3) for the *l*-derivative.

SUMMARY

The optical resolution of *dl*-menthol has been effected through its conjugation with glucuronic acid in the rabbit. Two methods were used:

(a) The ammonium salts of the *d*- and *l*-menthylglucuronides, excreted in the urine following the oral administration of *dl*-menthol, were fractionally crystallized.

(b) *l*-Menthol was eliminated by taking advantage of its greater ease of oxidation in the rabbit than *d*-menthol. By re-feeding menthol recovered from a previous administration of *dl*-menthol, nearly 90% of the *l*-menthol was oxidized biologically and the excreted glucuronide was mainly *d*-menthylglucuronide which was easily purified.

d-Menthol, from hydrolysis of *d*-menthylglucuronide, was obtained in about 10% yield, calculated on the *dl*-menthol fed. *l*-Menthol was not readily obtainable in optically pure form by these methods.

The excretion of conjugated menthol, following a dose of 3 g. per rabbit, is complete in about 30 hr., the material excreted throughout this period having a constant ratio of *d*- to *l*-menthol.

The following derivatives of *d*-menthol are described for the first time: *d*-menthylglucuronide and its ammonium salt, *d*-menthylphenylurethane and *d*-menthyl 3:5-dinitrobenzoate.

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CLXXXVII. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B₁-DEFICIENT STATES

IV. THE ACCUMULATION OF PYRUVIC ACID AND OTHER CARBONYL COMPOUNDS IN BERI-BERI AND THE EFFECT OF VITAMIN B₁

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RESULTS are reported in this paper on the amounts of bisulphite-binding substances (B.B.S.), particularly pyruvic acid, in blood, cerebro-spinal fluid, urine and milk obtained from adult subjects. Most of these subjects when investigated were in various stages of vitamin B₁ deficiency; for purposes of comparison, however, some observations have been made on apparently healthy persons. Comparative data have also been obtained by determining the pyruvate and B.B.S. levels after administration of vitamin B₁. Particularly striking results have followed the treatment of cases of fulminating beri-beri with pure vitamin B₁ (thiamin).

Until recently, little attention has been paid to the occurrence in body fluids of carbonyl compounds except the "ketone bodies"—acetone, acetoacetic acid and β -hydroxybutyric acid, which, when they accumulate in the blood, are considered to be responsible for the syndrome called "ketosis" or "ketonaemia". There is, however, a growing interest in such substances in relation to intermediate metabolism in tissues, especially evident in work on muscle, liver and brain, and attention is likely to turn more and more to the origin of metabolites found in unusual amounts in body fluids. Alterations in body fluids such as have been found in the body fluids in beri-beri ought always to be related to the metabolism of the cells of the body which are primarily affected by the vitamin deficiency. An important advance in our understanding of disease processes may be looked for when changes in body fluids are interpreted in terms of what may be called "the chemical pathology of the cell", in place of the conception of a "toxaemia" which is so readily and commonly invoked in medicine.

Observations on the occurrence of B.B.S. substances in body fluids include investigations on (a) pyruvic acid in normal and diabetic urine [Fricke, 1922; Pi-Suñer & Farran, 1936], in blood [Westerkamp, 1933] and mammalian sera [Mendel *et al.* 1931], in blood and urine after exercise [Johnson & Edwards, 1936]; (b) aldehydes in blood in disease [Stepp, 1920; Stepp & Lange, 1920; Stepp & Feulgen, 1921], including formaldehyde in blood [Stepp & Zumbusch, 1920], in urine [Stepp, 1922; Reisser, 1916], acetaldehyde in blood [Fabre, 1925; Gee & Chaikoff, 1926], in relation to avitaminoses [Kauffman-Cosla & Roche, 1927; Palladin & Utewski, 1928; Kauffman-Cosla *et al.* 1931; Handovsky, 1935], (no acetaldehyde could be detected in the urine of vitamin B₁-deficient rats [Simola, 1936]); (c) methylglyoxal in urine [Pi-Suñer & Farran, 1936] and

in relation to the toxic symptoms in vitamin B₁ deficiency [Vogt-Möller, 1931; Geiger & Rosenberg, 1933; Popoviciu & Munteanu, 1934] particularly in breast milk [Asakura, 1932; Takamatsu, 1934]; and (d) of α -ketoglutaric acid in blood and urine [Krebs, 1938] and in relation to vitamin B₁ deficiency [Simola, 1936; Krebs, 1937].

The preliminary observations of the occurrence in human subjects in acute beri-beri of an increase of 3–4 times the normal amount of B.B.S. in the body fluids and the isolation of the 2:4-dinitrophenylhydrazone of pyruvic acid from blood, urine and cerebro-spinal fluids have already been reported [Platt & Lu, 1935; 1936]. In a note [Johnson *et al.* 1935] on the level of carbonyl compounds in human blood, values were given showing that in a number of diseases (mainly mental and metabolic) there was no significant increase above the normal level (also determined) and it was considered that carbonyl compounds other than pyruvic acid, acetone and acetoacetic acid were likely to occur in normal blood. The isolation of pyruvic acid from the blood of B₁-deficient animals was reported later by Johnson [1936].

Platt & Lu [1935; 1936] found that in contrast with results from the blood of rats and pigeons, in man only a fraction of the increase of B.B.S. could be accounted for by the increase of pyruvic acid as measured by the hydrazone method. It was also appreciated that B.B.S. might accumulate in conditions other than beri-beri and, whilst normal values for these substances were found in a small group of patients with clinical conditions similar in some respects to beri-beri, it was stated, in view of the evidence of the peculiar relationship of pyruvic acid to metabolism in B₁-avitaminosis, that it might be necessary to search specially for pyruvic acid. Wilkins *et al.* [1937] and Taylor *et al.* [1937] have now found an elevation of B.B.S. in conditions associated with acidosis, ketosis, anoxaemia, "toxaemia" and uraemia as well as in vitamin B₁ deficiency. Wilson & Ghosh [1937] have published evidence of increased values in epidemic dropsy, but the contribution of pyruvic acid to these increases and the effect on them of vitamin B₁ has not yet been determined. Recently Wilkins *et al.* [1938] found values for pyruvic acid by the hydrazone method of only 0.46, 0.72, 0.73 mg./100 ml. in three cases of nutritional deficiency with B.B.S. values 9.6, 13.8 and 6.6 mg./100 ml. calculated as pyruvic acid. The results of Shindo [1937, 1] show increased B.B.S. in beri-beri and a fall in the amounts of these substances after giving vitamin B₁. He believed from the analysis of the hydrazone he isolated from blood that the substance accumulating was acetaldehyde; but later [Shindo, 1937, 2] he reported the isolation of pyruvic acid hydrazone.

Platt & Lu [1936] were able to relate the increase of B.B.S., including pyruvate, to vitamin B₁ by demonstrating that the levels of these substances dropped after the administration of concentrates of vitamin B₁. In July 1935, a "usually fatal" case (no. 2196) recovered after treatment with 750 I.U. of vitamin B₁ in the form of marmite and a fall was recorded of B.B.S., pyruvic acid and "methylglyoxal" in the blood and cerebro-spinal fluid as well as remission of some clinical signs of beri-beri. During the summer of 1936 and 1937, however, pure vitamin B₁ was obtained and its effects on some of the carbonyl compounds in body fluids are recorded in this paper. A number of clinical features of beri-beri have been linked up with biochemical disturbances. In a contribution to a recent discussion [Platt, 1938], it was briefly shown how variations in B.B.S. and pyruvic acid in the blood had led to the elucidation of some factors which appear to contribute to the development of beri-beri.

EXPERIMENTAL

All pyruvate estimations were carried out by the specific hydrazone method as previously described [Platt & Lu, 1936; Lu, 1939, 1]. The vitamin B₁ used for the injection was prepared as described in the following paper of this series.

The patients investigated all showed some evidences of deficiency of vitamin B₁ on clinical examination. For present purposes they have been divided into two groups: (1) a subacute form, and (2) an acute, fulminating form. The latter group comprises patients represented previously [Platt & Lu, 1936] as + + + + deficiency of vitamin B₁; no attempt is made to subdivide the remainder. The patients in the second group are best described as being the "usually fatal cases" before the introduction of potent preparations of vitamin B₁.

RESULTS

Pyruvic acid and B.B.S. in blood and cerebro-spinal fluid

Measurements have been made of the amount of pyruvic acid and B.B.S. in the blood and cerebro-spinal fluid of "normal" subjects (Table I).

Table I. *Summary of "normal" values for B.B.S. and pyruvic acid in blood and cerebro-spinal fluid*

	No. and nature of subjects	Values
mg. pyruvic acid in 100 g. blood	60 resting and "cured" (2-3 tests on each)	All between 0.4 and 0.75
mg. B.B.S. (calc. as pyruvic acid) in 100 g. blood	23 "apparently healthy" Chinese males [Platt & Lu, 1936]	(2.22-4.8) Mean 3.27; standard deviation 0.70
mg. pyruvic acid in 100 g. C.S.F.	12 after adequate dose of vitamin B ₁	3 < 0.4 6 were 0.4-0.55 3 were 0.70-0.75
mg. B.B.S. (calc. as pyruvic acid) in 100 g. C.S.F.	12 after vitamin B ₁	—

Table II. *Pyruvic acid and B.B.S. in blood and cerebro-spinal fluid in subacute and acute beri-beri*

	Subacute beri-beri					Acute beri-beri				
	No. of cases	Mean	Standard error of mean	Standard deviation	Range	No. of cases	Mean	Standard error of mean	Standard deviation	Range
mg. pyruvic acid per 100 g. blood	84	0.93	±0.043	0.40	0.17-1.93	38	2.72	±0.212	1.31	1.0 - 5.77
mg. B.B.S. (calc. as pyruvic acid) per 100 g. blood	65	4.5	±0.14	1.16	2.7 - 7.3	36	10.1	±0.49	2.93	5.8 - 16.3
mg. pyruvic acid per 100 g. C.S.F.	30	0.72	±0.038	0.21	0.41-1.32	29	1.69	±0.18	0.69	0.74- 3.28
mg. B.B.S. (calc. as pyruvic acid) per 100 g. C.S.F.	25	2.81	±0.15	0.73	1.8 - 4.2	25	3.7	±0.38	1.92	2.6 - 9.2

A summary of the values for levels of pyruvate and B.B.S. is given in Table II.

It is evident from the results in Tables I and II that (1) the values for pyruvate and B.B.S. in blood and cerebro-spinal fluid in subacute cases are within normal limits or only slightly raised, (2) there are striking increases found in acute beri-

beri and (3) the accumulation is more marked in the blood than in the cerebro-spinal fluid.

The association of the various raised levels is clearer from grouped results; for the subacute cases, these are given in Table III. In 85 subacute cases B.B.S. in blood has also been determined in 68, and the cerebro-spinal fluid examined for pyruvic acid in 30, for B.B.S. in 25 cases.

Table III. *Grouped results for subacute cases*

Blood pyruvic acid level mg./100 g.	No. of cases	B.B.S. in blood, mg./100 g. (calc. as pyruvic acid)				Pyruvic acid c.s.f. mg./100 g.			B.B.S. in c.s.f. (calc. as pyruvic acid) mg./100 g.
		2.5-	5-	7.5-	10-20	0.4-	0.6-	1.2	
0.2-0.6	19	15	4	—	—	2	—	—	4
0.6-1.0	31	19	5	—	—	6	9	1	12
1.0-2.0	35	13	7	4	1	—	10	2	9
		47	16	4	1	8	19	3	25
Totals	85	68				30			25

On account of the need for beginning treatment it was not always possible to secure specimens from these patients under basal metabolic conditions. Values for blood pyruvic acid between 0.6 and 1.0 mg. per 100 g. cannot therefore strictly be regarded as being associated with vitamin B₁ deficiency. In most cases, however, at least half an hour's rest in bed was allowed and it has been found (as will be reported in the following paper in this series) that this period is sufficient for the restoration to normal of increased levels of blood pyruvic acid in non-deficient subjects. Furthermore, when treatment had been withheld, patients with blood pyruvic acid levels of about 1 mg. per 100 g. maintained this increase for many hours under resting conditions until vitamin B₁ was administered. There is little doubt that in the third group, having 1-2 mg. per 100 g. pyruvic acid in the blood, there is an increase which is related to vitamin B₁ deficiency. It is probable that some of the cases included in the 0.6-1.0 mg. per 100 g. group have also increased values resulting from their deficiency in vitamin B₁. In the group with the higher levels it is clear that there is an increase of B.B.S.; this increase is more than can be accounted for by the accumulation of pyruvic acid. In cases with raised levels for pyruvate in the blood there is, in the cerebro-spinal fluid, an increase of pyruvate which, however, is not increased to the same level as that in the blood. All the values for B.B.S. in the cerebro-spinal fluid are below 4.5 mg./100 g. and there are a number between 5.0 and 10 mg./100 g.

In 38 cases of acute beri-beri, values for blood B.B.S. and for B.B.S. and pyruvate in cerebro-spinal fluid have been determined in a number of instances in the same sample of fluid. These are grouped for different values of blood pyruvate in Table IV A and B.

40% of these cases have values between 1 and 2 mg. per 100 g. pyruvic acid in the blood, the remainder are in groups ranging between 2 and 6 mg./100 g.

The numbers of cases in each group diminish as the value for the group increases. With increase in the level of the blood pyruvic acid, the B.B.S. in the blood and cerebro-spinal fluid and the pyruvic acid in the cerebro-spinal fluid tend to rise; the numbers of cases with these raised values also increase for the higher blood pyruvate groups.

There is a group with blood pyruvic acid values between 1 and 2 mg. per 100 g. in the series of both subacute and acute cases. It is clear from a comparison of the

Table IV A. *Acute beri-beri. Chemical and clinical data*

Record no.	B.B.S. mg./100 g.			Pyruvic acid, mg./100 g.			Methylglyoxal (qual. or mg./100 g.)		C.S.F. pressure mm. water	Blood pressure systolic/diastolic mm. Hg.	Comments
	Blood	C.S.F.	Urine	Blood	C.S.F.	Urine	Blood	C.S.F.			
1574	15.3	7.7	—	—	—	—	—	—	300	80/50	3 days acute phase; died 15 hr. after admission
1904	16.1	6.71	—	—	—	—	—	—	290	58/30	2 days acute phase; died 1½ hr. after admission
2081	10.9	3.86	—	—	—	—	—	—	—	70/40	—
2196	10.16	7.86	—	2.38	1.48	—	++	++	260	105/730	4 days acute phase; partly cured. See Fig. 1
2241	9.70	7.06	—	—	—	—	—	—	240	—	12 hr. acute phase; died
2472	12.3	10.1	—	6.15	4.2	—	—	—	Raised	—	Infantile beri-beri; died 2 hr. after admission
N.T.H. 1293	14.0	5.47	—	7.47	4.6	—	0.27	—	300	85/40	1 day acute phase; died 2 hr. after examination
2984	10.8	3.31	—	3.54	2.4	—	—	—	300	90/30	6 hr. acute phase; died 2 hr. after admission
3007	10.7	5.14	—	5.04	3.31	37.0	—	—	270	100/50	History not known; unusually marked improvement after venesection. Died 1½ hr.
3089	7.7	—	81.4	—	—	—	—	+	—	80/?	2 days acute phase; died 3 hr. after admission
3175	9.9	5.1	—	4.56	3.33	55.3	0	0.085	—	90/30	2-3 days acute phase; died 8½ hr. after admission. See Fig. 2
3485	11.18	8.2	21.6	1.29	1.92	—	++	—	—	—	A boy of 6 years (42 lb.) a complicated case; had intermittent pyrexia; evening rise to 103° F. See Fig. 3

Table IV B. *Grouped results for acute cases*

Blood pyruvic acid level mg./100 g.	No. of cases	B.B.S. in blood (calc. as pyruvic acid) mg./100 g.				Pyruvic acid in c.s.f. mg./100 g.				B.B.S. in c.s.f. (calc. as pyruvic acid) mg./100 g.			
		5-	7-	10	15-20	0.6	1	2-3	4	2.5	5	7.5	10
1-	15	5	5	4	—	3	8	1	—	8	3	—	—
2-	9	2	3	2	—	—	5	—	—	1	3	—	1
3-	7	—	1	4	—	—	3	3	—	2	2	—	2
4-	4	—	1	2	1	—	1	1	1	—	2	—	1
5-	3	—	3	—	—	—	—	2	—	—	1	—	—
	38	7	13	12	1	3	17	7	1	11	11	—	4
Total	38	33				28				26			

values recorded in Tables III and IV that there are increases of pyruvic acid and other carbonyl compounds in blood and cerebro-spinal fluid, more marked in the acute than in the subacute cases. Thus for example more than half of the values for B.B.S. in blood in the subacute series fall below 5.0 mg. per 100 g. while in the acute cases all values are above this level.

Pyruvic acid in urine in vitamin B₁ deficiency

The results of previous work on the occurrence of pyruvic acid in urine have yielded no conclusive results. Kendal & Friedmann [1930] found an "apparent" excess of pyruvic acid (4.5 and 3.9 mg./100 ml.) in morning urine and an apparent deficit in urines obtained after drinking large quantities of water. They were, however, unable to prove that these variations were really due to pyruvic acid. They state further that several workers, notably Fricke [1922], have stated that pyruvic acid may be found in urine. Reference to Fricke's original paper shows, however, that he failed to obtain any evidence of the presence of pyruvic acid in an attempt to isolate it as the phenylhydrazone. His method, moreover, was sensitive only to a concentration of 1 : 1000 and he pointed out that his inability to isolate the compound did not exclude its presence in urine.

Pyruvic acid was isolated as the 2:4-dinitrophenylhydrazone from the urine of patients with acute beri-beri [Platt & Lu, 1935; 1936]; values were obtained of 37.0 and 53.3 mg./l. For 25 apparently healthy Chinese males (college students) values for two samples of freshly passed early morning urines between 0.25 and 0.65 mg./100 ml. were obtained. Occasionally values of 0.7-1.25 mg./100 ml. were found; these on further investigation were attributable to athletic activities some hours before the test.

In a series of 7 healthy subjects on adequate intakes of vitamin B₁ observed over a period of 15-20 days, amounts of "pyruvic acid" by the hydrazone method ranging between 1.25 and 7.5 mg. per day were estimated. Amounts of 10-20 mg. "pyruvic acid" were found to be excreted by subjects in a state of frank vitamin B₁ deficiency.

An attempt was made to confirm the presence of pyruvic acid in urine from "normal" subjects by isolation of the hydrazone from a mixed batch of several litres of urine. A reddish brown preparation was obtained from which no single compound could be isolated. It is likely that there are substances in urine other than pyruvic acid which yield alkali-soluble 2:4-dinitrophenylhydrazones giving a colour like that of the pyruvic compound with alcoholic KOH, e.g. glucuronic acid [Case, 1932], acetoacetic acid [Clift & Cook, 1932], and α -ketoglutaric acid [Krebs, 1938]. Since the hydrazone method does not appear to be satisfactory

for estimating pyruvic acid in normal urine, detailed results obtained and further comment are omitted. The new micro-method recently described [Lu, 1939, 1] would appear to be better suited for further studies of this question.

Pyruvic acid in milk and the peroxidase reaction

It has been possible to obtain a few specimens of human milk for estimation of the pyruvic acid content. The results are given in Table V.

Table V. *Pyruvate in apparently healthy and vitamin B₁-deficient mothers' milk*

State of deficiency	No. of cases studied	mg./100 g. of milk	
		Range	Mean
Normal (Chinese)	8	0.1-0.24	0.16
	(11 tests)		
Normal (British)	3	0.12-0.26	0.18
Subacute (Chinese)	5	0.34-0.53	0.41

Tested for peroxidase with the modified guaiac reagent of Arakawa [1930] evidence of peroxidase activity was found in three or four of the first group and in none of four of the second group. The addition of pyruvic acid in amounts required to raise the value to 5 mg./ml. failed to inhibit the reaction in normal milk. In view of the results reported in a series of papers by Japanese workers (some references are given above) an attempt was made to detect the presence of methylglyoxal in milk in which no peroxidase reaction was obtained. A blue colour was obtained with alcoholic KOH with the supposed bis-hydrazone, but it could not accurately be matched against a standard prepared from pure methylglyoxal.

The Arakawa reaction carried out with cow's milk (diluted 1:10) to give an intensity similar to that with human milk was inhibited by the addition of milk from a wet nurse with vitamin B₁ deficiency.

In a further series of tests on a deficient subject seven values for pyruvic acid gave a mean of 0.49 mg./100 ml. (0.38-0.66); following the administration of 2 mg. of synthetic vitamin B₁ the value fell to 0.27 mg./100 ml. (see Table VI).

Table VI

Day of observation	Blood	Milk
1	0.96	0.51
2	1.01	0.38
3	0.84	0.46
4	—	0.47
5	—	—
6	0.73	0.66
7	—	0.57
8	0.61	0.39
9	—	0.27

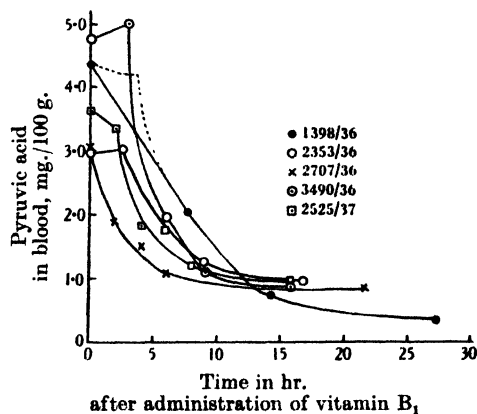
Note. 2 mg. vitamin B₁ given on the 7th day.

The effect of vitamin B₁ on pyruvate and B.B.S. in the blood, urine and cerebro-spinal fluid in fulminating beri-beri

In Table VII records of blood and cerebro-spinal fluid changes in four cases after treatment with vitamin B₁ are presented. These are chosen from a number of "usually fatal" cases which have been cured. It has been possible to make several estimations at intervals during recovery.

Table VII. *Pyruvic acid and B.B.S. in blood and cerebro-spinal fluid in fulminating beri-beri cases after treatment with vitamin B₁*

Case no.	Time	Blood, mg./100 g.		c.s.f., mg./100 g.	
		B.B.S.	Pyruvic acid	B.B.S.	Pyruvic acid
1398	2.20 a.m.	9.2	4.36	—	1.77
	10.00 a.m.	15.8	2.05	—	—
	4.30 p.m.	8.5	0.74	—	1.13
	5.30 a.m.	9.5	0.42	—	—
2353	9.00 p.m.	14.2	3.97	—	—
	11.40 p.m.	12.4	4.00	—	—
	6.25 a.m.	10.7	1.26	—	—
	3.00 p.m.	13.2	0.95	—	—
2707	12.00	12.2	3.05	8.1	2.34
	2.00 p.m.	11.0	1.84	—	—
	4.00 p.m.	9.8	1.47	—	—
	6.00 p.m.	9.6	1.02	—	—
	9.30 a.m.	8.31	0.84	—	—
2490	2.25 p.m.	11.2	4.76	6.5	2.52
	5.25 p.m.	13.0	5.04	—	—
	8.25 p.m.	7.7	1.94	—	—
	11.30 p.m.	6.3	1.10	—	—
	6.00 a.m.	5.3	0.89	—	—
	10.25 a.m.	5.0	0.47	—	—

Fig. 1. Blood pyruvate at intervals after treatment with vitamin B₁ in five cases of acute beri-beri.

The results of the blood pyruvate in these cases and that of case no. 2525/37 are plotted in Fig. 1 to show the "lag period".

The initial values for pyruvic acid lie roughly between 3 and 5 mg./100 g. of blood and in all cases they are substantially reduced to 1–2 mg./100 g. 5 hr. after treatment with vitamin B₁. Amounts of vitamin B₁, ranging from 40 to 5 mg. have been given to the different cases. The subject receiving 40 mg. dose showed a slightly more rapid fall of blood pyruvate than in the other cases. In three of the five cases given 5–10 mg. of vitamin B₁ (see Fig. 1) there appears to be a lag period in the restoration of normal values; the data are insufficient in case 1398/36 to determine the presence of a lag but there is a comparatively slow recovery. It is worth mentioning, in regard to the relationship of basic disturbances in cells as against alteration of metabolite levels in body fluids,

that in both cases (1398/36 and 3490/36) in which the urine content of "pyruvic acid" was determined, the levels are less directly related to the improvement in conditions as seen clinically than are the changes in the blood.

From purely clinical considerations it would appear that, in the absence of factors which maintain an increased requirement for vitamin B₁ in the recovery period, 5 mg. are an adequate curative dose for these severe cases. This fact is of much economic importance in countries where beri-beri is endemic and supplies of the vitamin may be low; especially as it has been stated in recent publications (e.g. Sinclair [1939]) that from 20 to 50 mg. are requisite. In instances in which administration of the vitamin failed to bring about a cure there were complications due to concurrent zymotic disease or else death ensued a short time after the treatment had been begun. From the data given several hours would be expected to elapse before conditions approaching normal are established. There appears to be no important difference in the efficiency of the synthetic as compared with the natural crystalline product; a similar result has been obtained for animals by Eckler & Chen [1937]. No untoward effects are observed as a result of giving the vitamin by the intravenous rather than other recommended routes.

DISCUSSION

Raised levels of pyruvate in body fluids

From the data presented above it is clear that increased amounts of pyruvate are found in all cases of fulminating and in a number of cases of subacute beri-beri. Alterations of pyruvate levels in body fluids are no doubt mainly, if not entirely, secondary to an altered chemical state of such tissues (for review of work on tissue changes see Peters & Sinclair [1933]) which require vitamin B₁ for the complete and normal metabolism of pyruvic acid. It will be necessary to investigate the changes in the affected tissues by a direct method before a satisfactory correlation is possible. De Jong [1936] was unable to establish a direct relationship between the clinical changes in vitamin B₁ deficiency conditions and B.B.S. values in the blood for the reason, no doubt, that the blood changes are only a reflexion of pathological disturbances in the cells primarily affected.

Pyruvate in the body does not appear to be toxic in the amounts in which it accumulates in beri-beri [see Lu, 1939, 2]. Thus there is no record of ill-effects in man when levels were raised by intravenous injection or oral administration of Na pyruvate [Wilkins *et al.* 1938; 1939]. Flock *et al.* [1938] report recovery of dogs when their blood levels increased to as much as 70 mg./100 ml. (except when a deleterious alkalosis supervened). Recovery takes place after exercise (see the following paper of this series) even when blood levels similar to those which have been found in acute beri-beri are reached. On the other hand Kermack *et al.* [1927] found a toxic effect of Na pyruvate on injection in rabbits: this, however, may be related to the presence of some toxic substance which is known [Lipschitz *et al.* 1938] to develop in concentrated pyruvic acid solutions after standing.

The possibility must be recognized that pyruvate accumulation is specific for vitamin B₁ deficiency, only in so far as, in vitamin B₁ deficiency, intermediate carbohydrate metabolism is deranged. Also, the interpretation of blood levels may be complicated because of an increase of the metabolites accumulated arising as a result of impaired kidney function, which is known to manifest itself in acute beri-beri as an increase of non-protein nitrogen levels [Platt & Lu, 1936] and possibly as a phosphate retention. Further complications

arise in consequence of the disturbance of metabolism associated with heart failure which contributes to the beri-beri syndrome. The occurrence of creatinuria [Kindler, 1936] in heart failure is of interest in this connexion for it was on account of an observation that increased amounts of creatine were to be found in the urine from patients with beri-beri that attention was first directed to this disease and to an investigation of changes then supposed to be disturbances of metabolism in muscle due to vitamin B₁ deficiency.

Increases of other B.B.S.

Increases of B.B.S. have been observed to be greater than can be accounted for by the increase of pyruvic acid. Furthermore, it is often found that pyruvic acid may be restored to normal levels in the blood while at the same time there is only a slight reduction in the amount of B.B.S. The first question that arises is as to the nature of these substances. From the available evidence acetaldehyde, methylglyoxal and α -ketoglutaric acid have to be included in this group; two other substances which may be mentioned as being of interest in future investigation are glyceraldehyde and dihydroxyacetone. There are at least three possibilities as to their origin. They may be products of deranged metabolism due solely to insufficient amounts of vitamin B₁; they may be derived from pyruvic acid or, thirdly, they may be the products of some associated deficiency or other metabolic error.

In pure vitamin B₁ deficiency produced experimentally in animals it is significant that almost all of the increase of B.B.S. is accounted for by accumulation of pyruvic acid [Thompson & Johnson, 1935]. It appears then, that unless intermediate metabolism in rats and pigeons has important differences from that in man in this respect, the accumulation of B.B.S. other than pyruvic acid is not a direct result of vitamin B₁ deficiency.

The second possibility, that B.B.S. may be formed secondarily from pyruvic acid, has been investigated from time to time [Embden & Oppenheimer, 1912; Annau, 1934; Krebs & Johnson, 1937] and it is known that pyruvic acid can be converted into ketones. The results of Lu (unpublished) on rat and rabbit blood and the results of Wilkins *et al.* [1938] using human blood showed that pyruvate added to blood before or after it was withdrawn from the body was converted into other ketones which in turn are more slowly metabolized. Some years ago Stepp & Behrens [1923] suggested that pyruvic acid was converted by a carboxylase in the blood into acetaldehyde. Later, Simola [1932] claimed to have shown that there was a considerable reduction in the amount of cocarboxylase in the liver and brain of rats after being 30 days on the vitamin B₁-deficient diet. While these results have not been fully confirmed [Lipschitz *et al.* 1938], other measurements of the amounts of cocarboxylase in the tissues of normal and B₁-avitaminous animals have been reported [Ochoa & Peters, 1938] in which diminished amounts of cocarboxylase were found in vitamin B₁ deficiency. Considerable interest in cocarboxylase has developed since Lohmann & Schuster [1937] showed that vitamin B₁ pyrophosphoric ester is the cocarboxylase of alcoholic fermentation by yeast, pyruvic acid being converted into acetaldehyde. In view of these observations and of the occasional reports that acetaldehyde occurs in body fluids it appears that the problem is worth reinvestigation. Lipschitz *et al.* [1938] state that it is not impossible that acetaldehyde is still an intermediate in the dismutation of pyruvic acid in which acetic acid is formed. It should be noted that Briggs [1926] was unable to obtain evidence of conversion into acetaldehyde of pyruvate injected into normal animals; this, however, cannot

be accepted as evidence against the possibility of such a change in vitamin B₁ deficiency.

The third group of possible explanations involves a consideration of the association of other metabolic and nutritional defects. The fasting state is one which needs to be considered. Lipschitz *et al.* have discussed this in relation to delayed removal of pyruvate and have drawn attention to the work of Weil-Malherbe [1937] in support of their contention that other intermediates in carbohydrate metabolism appear to be required to assist in the removal of pyruvate. Considerable increases in B.B.S. in the blood are reported by Wilkins *et al.* [1938] in subjects with a "nutritional deficiency" but who show no increase of blood pyruvate. From reports of the type of subject with which they are working it is likely that in addition to being alcoholics their patients had diets with a higher fat/carbohydrate ratio than ours. It is conceivable that in these circumstances there might be a relative increase of B.B.S. to pyruvic acid in the blood.

An associated insufficiency of glutathione may, according to the work of Gaddie & Stewart [1935], account for the appearance of methylglyoxal which may be one of the substances contributing to the total B.B.S.

Simola [1936] suggested "that at least two components of the B group play an important part in the normal keto-acid metabolism". He found that the excretion of α -ketoglutaric acid in the urine occurred when experimental animals were kept on a diet deficient in all but vitamin B₁ of the B complex. This observation is of particular interest in relation to the observation that vitamin deficiency states as they are seen in China are almost always multiple, especially when their manifestations are of a minor type.

Methylglyoxal needs further consideration. Meyerhof [1934] considers the occurrence of this substance in muscle metabolism to be an artefact, the substance being produced by decomposition (not involving an enzymic mechanism) from triosephosphate. If this interpretation is applicable to our findings then interest is shifted to a study of the accumulation of the precursor of methylglyoxal for, as has been shown, the reaction for this substance is obtained in our beri-beri cases and is no longer detected after treatment with adequate amounts of vitamin B₁. It is unlikely in this instance that the explanation put forward by Müller [1933] that the "methylglyoxal" reaction is due to a trace of glucose is adequate. An attempt to determine the occurrence of a phosphorylated precursor of methylglyoxal in the blood was complicated by the fact that the involvement of the kidney in beri-beri appeared to have led to a retention of phosphates. There is evidence that methylglyoxal, if present in increased amounts, may have toxic effects [Sjollem & Seakle, 1926; Fischler, 1927; Kermack *et al.* 1927; Takamatsu & Sato, 1934].

The behaviour of "blood sugar", lactic acid and other related substances is of importance but is not discussed further here as the scope of this study has been limited to a consideration of bisulphite-binding carbonyl compounds (glucose has some capacity for binding bisulphite [Clift & Cook, 1932] but only under conditions different from those in the method of estimation employed).

The effects of administration of vitamin B₁

The above results show a marked effect of vitamin B₁ on pyruvate levels in body fluids; there is a reduction of the levels in blood and cerebro-spinal fluids and increased urinary output of pyruvate some time after injection. There is a lag in the fall of the pyruvate after amounts of 5-10 mg. of vitamin B₁. This lag may not accurately represent the changes actually occurring in the affected

tissues, which must be examined directly before the nature of the lag can be determined. The B.B.S. are not so readily restored to normal levels after vitamin B₁ as pyruvate; further investigation is required before this can be explained. As regards the lag just referred to, it is probable that vitamin B₁ has to undergo certain chemical changes (e.g. phosphorylation or combination with a protein) before it can exert its activity. Banga *et al.* [1939] have indeed shown, contrary to what was formerly thought, that the "catatorulin effect" of cocarboxylase is the same as that of the free vitamin.

SUMMARY

1. The results of a comparative statistical study of the B.B.S. and pyruvate in the blood, milk and urine, of normal and vitamin B₁-deficient human beings, are given. The pyruvate level in normal human blood is of the order of 0.5 mg./100 g.

2. Maintained raised levels of blood pyruvate seem to be quite specifically associated with vitamin B₁ deficiency.

3. In the rapidly developing fulminatory types of beri-beri the initial level of blood pyruvate is a definite indication of the degree of the deficiency.

4. Several hours are required for the removal of accumulated pyruvate after administration of vitamin B₁.

5. About 5 mg. of the pure crystalline vitamin are needed in the acute cases to restore normal conditions with respect to pyruvic acid.

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CLXXXVIII. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B₁-DEFICIENT STATES

V. THE EFFECT OF EXERCISE ON BLOOD PYRUVATE IN VITAMIN B₁ DEFICIENCY IN MAN

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FOLLOWING the establishment [Platt & Lu, 1939] of the occurrence of increased amounts of pyruvic acid in the body fluids of patients suffering from beri-beri and the studies of Lu [1939] on the effect of muscular activity on the blood pyruvate of the rabbit, it appeared desirable that an investigation should be made of the relationships between pyruvic acid accumulation in the blood, amount of exercise and nutrition with respect to vitamin B₁; this has been carried out and the results are presented in this paper. There are a number of factors, of which increased muscular effort is one, which appear to contribute to the development of vitamin B₁ deficiency in man [Yang & Huang, 1934].

METHODS

The methods used for the estimation of pyruvic acid and bisulphite-binding substances (B.B.S.) have already been described [Platt & Lu, 1936; Lu, 1939].

Vitamin B₁ used for injection was made up (in a concentration of 1 mg./ml.) from the pure crystalline product in *N*/1000 HCl containing 20 % by volume of ethyl alcohol [Kinnersley *et al.* 1933]. The exercise consisted either of (1) sitting up and lying down in bed without assistance 20 times, taking approximately 45 sec., or (2) climbing 100 steps five times, the test occupying 8-10 min., of which about half was occupied in climbing. Blood was taken for analysis (a) at rest, (b) immediately after exercise and (c) at the end of half an hour's rest after exercise.

RESULTS

Some of the results obtained from experiments on the effects of exercise on blood pyruvic acid and B.B.S. in cured and deficient cases are collected in the following tables.

It will be seen from the tabulated results in Tables I and II that the B.B.S. values give no consistent changes after slight exercise, while the pyruvate values of the deficient cases maintained a higher level after $\frac{1}{2}$ hr. as contrasted with the cured cases. This will appear more clearly from the detailed accounts of two typical examples given below.

Fig. 1 gives results on an acute type of beri-beri case with subacute manifestations of deficiency (oedema of legs and feet, dyspnoea and palpitation on exertion) for 2 months, worsening 2 days before admission to the fulminating form (described as + + + + by Platt & Lu [1936]). After taking blood for

Table I. *Cured cases*

Hospital no.	B.B.S. (expressed as pyruvic acid) mg./100 g. blood			Pyruvic acid mg./100 g. blood		
	Immediately			Immediately		
	Initial level	after exercise	$\frac{1}{2}$ hr. later	Initial level	after exercise	$\frac{1}{2}$ hr. later
1859	4.7	4.7	4.2	0.86	0.90	0.81
2115	3.4	4.8	3.5	0.40	0.60	0.53
2574	3.5	4.3	3.5	0.57	0.71	0.47
2910 ₁	5.9	6.7	3.2	0.02	1.13	0.53
2910 ₂	3.3	3.6	2.9	0.72	0.65	0.40
1936	2.6	3.3	2.3	0.40	1.01	0.58
2936	3.2	3.7	3.5	0.48	0.91	0.49
3127	2.9	3.7	2.8	0.54	0.76	0.45
3180	4.2	5.7	4.4	0.45	0.90	0.50
3198	3.5	3.8	—	0.63	0.84	0.61
3212	2.6	2.8	2.9	0.54	0.54	0.60
3212	3.7	3.2	2.6	0.79	0.87	0.71
3252	3.1	4.8	4.4	0.73	0.81	0.72
3319	3.0	3.4	3.0	0.40	0.50	0.40
3321	3.4	3.6	2.6	0.45	0.67	0.59

Table II. *Subacute cases—before and after treatment with vitamin B₁*

Hospital no.	B.B.S. (expressed as pyruvic acid) mg./100 g. blood			Pyruvic acid mg. 100 g. blood		
	Immediately			Immediately		
	Initial level	after exercise	$\frac{1}{2}$ hr. later	Initial level	after exercise	$\frac{1}{2}$ hr. later
2707	2.6	3.4	4.30	0.59	0.74	0.88
2707	2.7	3.0	4.5	0.56	0.60	0.54
3012	2.6	3.1	3.4	0.71	1.02	1.00
3012	4.1	4.3	4.6	0.64	1.23	0.63
3146	3.9	5.4	5.4	0.94	1.10	1.47
3146	4.8	3.6	3.8	0.52	0.52	0.43
3146	3.6	3.5	4.4	0.66	0.68	1.52
3142	3.4	4.6	3.6	0.46	0.97	1.02
3142	3.1	4.2	3.7	0.91	1.05	0.58
3198	2.8	2.7	3.2	0.83	0.96	0.90
3198	2.7	3.0	3.5	1.50	0.52	0.60

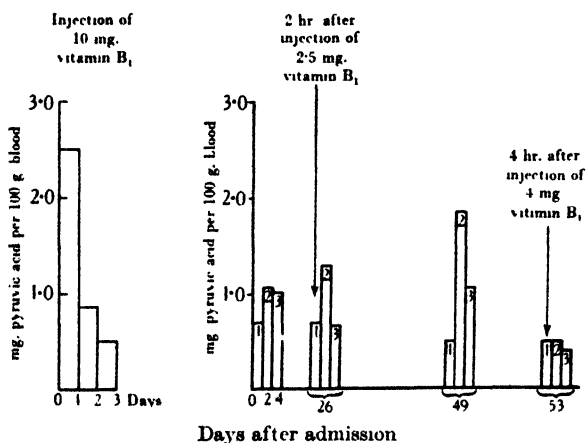


Fig. 1. Case 3012. 1, Resting level; 2, immediately after exercise; 3, 30 min. later.

analyses, 10 mg. of crystalline vitamin B₁ were given intravenously. The patient recovered, the blood pyruvic acid level being restored to normal. He was put on to ordinary hospital diet, about 70 % of the calorie value of which was taken in the form of highly milled rice; the estimated intake of vitamin B₁ being about 0.5 mg. or less per day.

A tolerance test to exercise in bed was made on the 24th day after treatment with vitamin B₁. An increase of pyruvate after exercise was found to occur and to be maintained after half an hour. Two days later a similar amount of exercise was given 2 hr. after a dose of 2.5 mg. vitamin B₁. There was in this instance an increase of pyruvic acid immediately after exercise but the value returned to the original resting level at the end of a further half hour. After another period of 23 days on the vitamin-poor diet the test was repeated. There was a definite rise in blood pyruvate after exercise and the resting level was not restored at the end of half an hour. This is in contrast with the result of a repetition of the test 4 days later when 4 mg. vitamin B₁ were given 2 hr. before the exercise; there was a low resting level, and no rise of pyruvate either immediately or half an hour after exercise.

Subject no. 3198/36 had a history similar to the foregoing. A test of his response to exercise was made on the 12th day after treatment with 10 mg. of vitamin B₁. Only a slight increase of pyruvic acid in the blood was noted after exercise and the initial value was attained after the rest period. The "resting" level was found to be slightly raised on the 19th day under observation; an insignificant increase followed exercise in bed. It may be noted here that this type of response is commonly obtained in the subacute type of beri-beri and that after a dose of 4-5 mg. of vitamin B₁ it is usual to find a lowered resting value and little or no increase after exercise.

This patient, on his 42nd day in hospital was subjected to the stair-climbing test. The first set of 100 steps he completed in 45 sec., the second required 95 sec., and only 80 steps of the fourth set were climbed in 2 min. 47 sec. The subject complained of weakness of the legs, the pulse rate increased from a resting value of 70 beats per min. to 110; he was distressed, cyanosed and in general presented the appearance of a man in the fulminatory stage of beri-beri. The blood pressure before exercise was: systolic, 110, diastolic, 80 mm. After exercise: 10.30 a.m., 104/68; 10.40, 70/40; 10.45, 80/60; 11.40, 90/60 and 14.00, 95/50. The pulse rate fell to 88 per min. at 14.00 and then gradually fell to its original level in the course of the following 24 hr.

The pyruvic acid in the blood rose to nearly 2 mg./100 g. immediately after exercise and 15 min. later had reached a level which is characteristic of the cases of beri-beri of the most acute and "usually fatal" type described in the previous paper of this series. The second point of interest is that 1 hr. later the pyruvate was still present in the blood in considerably increased amounts; a normal value was reached some 4 hr. after the exercise. It will be seen from Table III that the B.B.S. increased to 11.6 in the acute deficiency state and to 8.4-9.9 mg./100 g. after exercise; these increases are far in excess of the amount accounted for by the accumulation of pyruvic acid (see Fig. 2).

380 ml. of urine obtained immediately before the exercise began contained 0.2 mg. pyruvic acid per 100 ml.: a specimen (390 ml.) collected at 14.15 had 1.76 mg./100 ml.

Similar exercise tests were carried out on two normal men (laboratory assistants) and three cured patients who received 2.5 mg. of vitamin B₁ 4 hr. before the test. The blood pyruvate value showed no appreciable increase in any of these subjects after exercise.

Table III. *B.B.S. and pyruvic acid*

Case no. 3198

	mg./100 g. blood	
	B.B.S.	Pyruvic acid
During B ₁ -hypovitaminosis		
On admission	11.6	3.8
1st day after B ₁ treatment	7.6	1.33
2nd day after B ₁ treatment	4.6	0.56
3rd day after B ₁ treatment	6.5	0.46
After severe exercise tolerance test		
Resting cured level	3.7	0.47
Immediately after severe exercise	8.4	1.86
$\frac{1}{2}$ hr. later	9.9	3.56
1 $\frac{1}{2}$ hr. later	—	2.56
4 $\frac{1}{2}$ hr. later	4.1	0.62

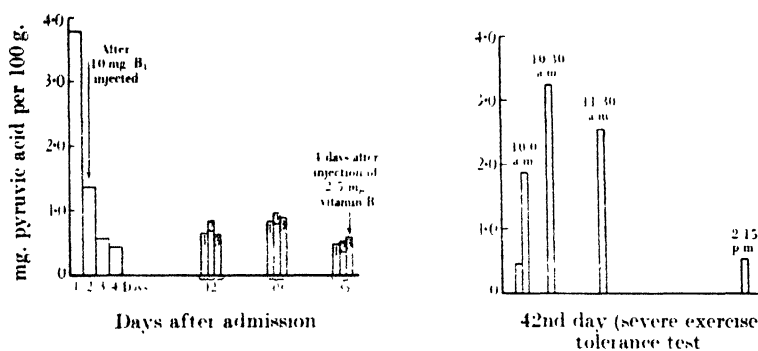


Fig. 2. Case 3198.

DISCUSSION

It has been shown in the previous paper of this series that when the urine of normal Chinese subjects contained increased amounts of "pyruvic acid" they had been engaged in some form of strenuous exercise. A similar observation had been reported by Johnson & Edwards [1937], who found that in normal subjects exercised to exhaustion pyruvic acid appears in the blood in amounts similar to those which are reached in severe beri-beri. Thus it is clear that it is possible to produce a state of exhaustion and a high level of pyruvic acid in the blood by an amount of muscular effort (stair-climbing) less than that which is well tolerated by subjects not deficient in vitamin B₁ and which can be accomplished without significant changes in the amount of pyruvic acid in the blood.

Another difference between the pyruvic acid change in the normal as compared with the vitamin B₁-deficient subject is in the time taken for the removal of the pyruvic acid which is produced during the exercise. In the experiments of Johnson & Edwards "normal" values were observed after less than 1 hr., whereas in the example described above, the high level of blood pyruvate is still maintained at the end of 1 hr. rest after the work is performed.

It may be mentioned here that the highest blood level is not immediately attained, no doubt for the reason that some time is taken for the metabolites to reach the blood stream from the cells in which they are formed. In B₁-deficient patients an amount of pyruvic acid greater than that found immediately after exercise has frequently been found in a test taken at the end of half an hour's rest following light exercise in bed. The need for time for products of

metabolism to reach the blood stream in B_1 -hypovitaminosis may afford the basis of explanation for the fact that de Jong [1936] was unable to correlate the occurrence of clinical phenomena with changes in B.B.S. in the blood of vitamin B_1 -deficient pigeons. It may also be noted that he did not find any increase of B.B.S. in his birds on "rousing" (exercising) them. An account of the blood pyruvate changes after exercise in normal rabbits has been given in a previous paper [Lu, 1939].

Results similar to those presented above were obtained for lactic acid by Inawashiro & Hayasaka [1928] for patients with beri-beri and for dogs with vitamin B_1 deficiency. They found that recovery was more prolonged in beri-beri than in the normal after exercise; that quite light exercise in the dog maintained a raised lactic acid level for long periods and that Na lactate injected into patients with beri-beri was synthesized to glycogen at much slower rates in the deficient as compared with the normal subject. Fisher [1931] also found that there was delayed removal of lactic acid from the heart and muscles of deficient pigeons after exercise. Lipschitz *et al.* [1938] have shown that polyneuritic birds are unable to remove injected pyruvate from the blood stream as rapidly as normal birds.

It has been demonstrated [Cowgill *et al.* 1931] that dogs which are exercised may be rendered deficient in vitamin B_1 in one-third to half the time required for those not exercised. Statements have been made to the effect that birds which have been rendered deficient have suddenly shown signs of the onset of the vitamin B_1 -deficiency syndrome when movements are attempted on being set free. Ariyama [1931] has claimed to have shown by experiments on pigeons that vitamin B_1 can diminish exhaustion and promote recovery from fatigue. There is abundant evidence in the clinical literature on the aetiology of beri-beri [cf. Shimozona, 1931] that muscular effort may contribute to the onset of beri-beri and that in a given deficient subject a lesion may be related to the amount of work performed by a particular part of the body. It is probable that increased muscular work is only one of a number of factors belonging to a group which have in common the property of increasing the requirement for, or deficiency of, vitamin B_1 on account of their contribution to the increase of metabolic activities in which this food factor is involved. The view has been expressed [Platt, 1938] that, given vitamin B_1 deficiency, the clinical manifestations of the state may be determined more by the operation of stress factors than by differences in intake of vitamin B_1 .

A practical issue of this investigation is the use which can be made of exercise to reveal latent vitamin B_1 deficiency states. The points which appear to be of most importance in designing a test are: (1) much less muscular effort is required to effect an appreciable increase of blood pyruvate in a vitamin B_1 -deficient subject than in one normally nourished with vitamin B_1 , (2) there is delayed removal of pyruvate from the blood after exercise during vitamin B_1 deficiency, and (3) the type of pyruvate response to exercise followed by dosage with vitamin B_1 also differs. Exercise tests may, however, be limited by the patient's inability to engage in heavy effort and it is sometimes undesirable that the patient be subjected to physical stress. In view of this it is suggested that a modification might be based on the determination of blood changes following a measured amount of work in a limb in which venous return is obstructed. In view of the possible association of fatigue, vitamin B_1 deficiency and exercise [Platt & Gin, 1934] this test might well be combined with observations of the development of sensations of pain in the exercised limb.

SUMMARY

1. Light muscular work by vitamin B₁-deficient human subjects is followed by an increase of blood pyruvate as measured by the 2:4-dinitrophenylhydrazone method. The rise of pyruvate may be maintained or still further increased at the end of half an hour's rest after the exercise.

2. After the intravenous administration of pure vitamin B₁, light exercise may or may not be followed by an immediate increase of blood pyruvate; following half an hour's rest after the exercise "resting levels" are found. This restoration of the level may be accounted for on the grounds that less pyruvate has been formed in exercise or that it has been more rapidly removed.

3. When the changes of blood pyruvate are inconsiderable, marked increase, accompanied by clinical manifestations resembling those of fulminating beri-beri, may follow heavier work. Still heavier work may, however, be well tolerated by subjects adequately supplied with vitamin B₁ and be insufficient appreciably to raise the blood pyruvate.

4. Values for blood pyruvate as high as those found in fulminating beri-beri may be attained after exercise of deficient subjects.

5. Similar high values have been observed by other workers to be reached by normal subjects when exercised to exhaustion. During rest after exercise, however, initial values are restored to normal subjects in less than 1 hr.

6. In vitamin B₁-deficient subjects there is delayed recovery after high values following exercise.

7. In an experiment in which high levels of blood pyruvate were produced in a deficient subject it was found that maximal values were not attained until some time after the completion of the exercise.

8. Bisulphite-binding substances (calculated as pyruvic acid) are increased when blood pyruvate is markedly increased, but to an extent greater than can be accounted for by the increase of pyruvic acid as estimated by the hydrazone method.

9. "Pyruvic acid" is excreted in increased amounts after exercise.

10. Evidences are recorded of circulatory changes accompanying accumulation of pyruvate after exercise of a deficient subject.

11. Excessive muscular work is discussed as one of a number of factors which may be responsible for the development of vitamin B₁ deficiency.

12. Exercise of the whole body combined with measurements of blood pyruvate may be used as a method for revealing latent vitamin B₁ deficiency.

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CLXXXIX. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B₁-DEFICIENT STATES

VI. THE FATE OF INJECTED PYRUVATE IN THE NORMAL RABBIT

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THE results of *in vitro* work have led to the postulation of several possible paths of pyruvate removal in animal tissues; namely (1) the scheme of Toenniesen & Brinkmann [1930] whereby 2 mol. of pyruvic acid are reduced to form 1 mol. of succinic acid, and the latter is then converted successively into fumaric, malic, oxaloacetic and 1 mol. of pyruvic acid, (2) the scheme of Krebs & Johnson [1937] whereby the oxidation of pyruvate is preceded by an anaerobic change: 2 mol. of pyruvate interact to form 1 mol. each of lactic acid, acetic acid and CO₂, (3) the oxido-reduction between pyruvate and an aldehyde, such as triosephosphate, giving lactic acid and an acid such as phosphoglyceric acid, (4) a direct oxidation like that which exists in the brain [Peters, 1936; 1938; Ochoa & Peters, 1938]. It is also relevant that according to Weil-Malherbe [1937] pyruvic acid is removed more effectively from brain slices in the presence of glucose than in its absence. However, little is known about pyruvic acid metabolism *in vivo*. The object of the following experiments is to throw some light on these questions.

Rabbits were used in most of the experiments. Instead of estimating the changes following administration of pyruvate in the blood, urine or excreta after some time, it was decided to load the rabbit suddenly with a definite amount of pyruvate by intravenous injection lasting for 1–2 min. and study the chemical changes resulting from this immediately after the injection, a few minutes later and in some instances 35 min. later, when the blood pyruvate level had returned to the pre-injection level.

Experimental details

All the rabbits were kept under resting conditions for at least 1 hr. before use, as previously described. Anaesthesia was begun by first giving a sedative dose of "Numal Roche" (1 ml. of 10 % solution per kg. body weight) and completed by using small amounts of ether. By the combined use of these two substances there was usually no struggle throughout the experimental period; and such methods of anaesthesia have been shown to cause no carbohydrate breakdown [see e.g. Sacks & Sacks, 1933].

Both gastrocnemius muscles of the rabbit were carefully dissected free from connective tissue, leaving the blood and nerve supply intact. One muscle was quickly excised in the resting state, and immediately dipped in liquid air to freeze. Pyruvate was then injected intravenously through the ear vein. Blood samples were taken from the opposite ear. After allowing the time necessary for the metabolism to take place the second gastrocnemius muscle (in the meantime

covered up with its own skin) was quickly excised and immediately dropped into liquid air. About 2-3 sec. elapsed between the excision and the time when the muscle was fully frozen. It never took more than 15 sec. to get the whole gastrocnemius frozen.¹ After the muscle had become very brittle, it was removed to a chilled mortar containing powdered CO₂ snow and ground to a fine powder. The mixture was then transferred to a 100 ml. weighing bottle containing 25 ml. of 5% CCl₃COOH (which was carefully weighed and cooled beforehand) to remove proteins. It was shaken frequently to ensure complete precipitation and extraction. The exact amount of muscle in each specimen was found by weighing the bottle again after room temperature had been reached. The precipitate was filtered and washed twice with 5 ml. 5% CCl₃COOH and the total volume diluted to 40 ml. Aliquot parts were taken for the various estimations. For the estimation of pyruvic acid the micro-method [Lu, 1939, 1] was used and for lactic acid the method of Friedemann & Graesser [1935]; the error caused by using trichloroacetic acid extract in the lactic acid estimations was checked with Zn lactate controls, and found to be negligible under the conditions described. The total reducing sugar in blood was estimated by the method of Folin & Wu [1920] and fermentable sugar, using the same technique except that the proteins were precipitated by 7% CuSO₄ and 10% Na tungstate. For the inorganic phosphate the method of Fiske & Subbarow [1925] was used. The triosephosphate and phosphopyruvate were estimated according to the method of Meyerhof & Lohmann [1934, 1, 2]. With the aid of the photoelectric colorimeter for estimation of small amounts of phosphate and pyruvate it was possible to make all the determinations on the extract from a single gastrocnemius muscle.

The pyruvate used for injection was prepared in the same manner as described previously [Lu, 1939, 1].

RESULTS

Some of the results are given in the following tables. It will be seen from the examples given in Table I that intravenous injection of small doses of pyruvate into rabbits gives rise first to a temporary increase of non-glucose substance (bisulphite-binding substance or B.B.S.) with a simultaneous increase of lactic acid; then a more marked increase of "fermentable sugar" occurs as the non-glucose reducing substances, lactic acid and pyruvic acid levels return to the resting values.

Table I. *Blood changes resulting from injection of small doses of pyruvate*

		All results are expressed as mg. per 100 g.				
Rabbit no.		Total reducing sugar	Fermentable sugar	Non-glucose substances	Pyruvic acid	Lactic acid
1 150 mg. pyruvic acid injected intrav.	Resting sample	129	104.2	24.8	0.84	28.8
	Immediately after injection	151.8	112.7	39.2	2.16	34.6
	8 min. later	160.8	128.0	32.8	1.51	38.6
	20 min. later	164.0	143.7	20.3	0.91	31.0
	30 min. later	170.0	148.6	21.4	0.90	28.4
2 200 mg. pyruvic acid injected intrav.	Resting sample	103.0	86.2	17.1	1.09	19.0
	Immediately after injection	113.0	88.4	29.6	3.08	20.4
	8 min. later	122.7	105.4	17.4	1.63	24.8
	20 min. later	137.9	118.3	19.6	1.10	21.6
	30 min. later	129.0	111.1	17.9	1.12	21.8

¹ This method instead of the technique of freezing *in situ* was adopted to eliminate interference with the circulation, since pyruvate was injected after excision of the control muscle.

Table II. *Pyruvate and lactate changes in blood*

Body wt. kg.	Pyruvic acid injected mg.	Total* blood g.	Pyruvic acid in blood, mg. per 100 g.		Increase % of pyruvic acid injected	Lactic acid in blood, mg. per 100 g.			Increase % of pyruvic acid injected
			Resting	After injection		Resting	After injection	Increase	
2.020	450	172	0.925	9.22 (9)	3.33	16	39 (9)	23	9.3
1.620	450	148	1.792	27.25 (4)	8.38	19.8	45.8 (4)	26	8.1
1.950	450	166	0.828	9.887 (8)	1.29	—	—	—	—
1.750	340	149	1.082	6.27 (8)	5.19	53.1	72.8 (8)	29.8	13.0
2.650	510	216	1.025	11.68 (8)	4.46	52.1	90.2 (8)	38.1	16.2
2.300	455	187	—	—	—	26.0	50.0 (14)	24.0	9.8
2.770	540	236	—	—	—	45.8	80.0 (5)	34.2	14.9
2.010	420	170	—	—	—	66.6	93.7 (8)	27.1	10.9

* Total blood was calculated by using the value 85 g./kg. body weight.

The numbers inside the brackets denote the time in minutes after the injection when the sample was obtained.

Table III. *Pyruvate and lactic acid changes in muscles*

Body wt. kg.	Pyruvic acid injected mg.	Total* muscle g.	Pyruvic acid in muscle, mg. per 100 g.		Increase % of pyruvic acid injected	Lactic acid in muscle, mg. per 100 g.			Increase % of pyruvic acid injected
			Resting	After injection		Resting	After injection	Change	
2.020	450†	470	0.908	3.06 (8)	2.07	33	60 (8)	+27	28.3
1.620	450	377	2.142	8.778 (4)	6.636	74	74 (4)	—	—
1.950	450	452	1.017	3.525 (8)	2.51	53	45 (8)	- 8	—
1.750	340	407	1.100	3.046 (4)	1.946	31	60 (4)	+29	34.8
2.650	510	616	1.050	3.935 (8)	1.885	59	53 (8)	- 6	—
2.200	455	510	1.177	2.305 (14)	1.125	46	52 (8)	+ 6	—
2.770	540	467	—	—	—	57	57 (8)	—	—

* Total muscle was calculated by dividing the body weight by 4.3 as found by actual dissecting and weighing of the muscle of two rabbits.

† Injection of 16 ml. of 0.9% NaCl alone did not alter the pyruvate and lactate levels in the blood or the muscles.

It has been shown [Lu, 1939, 2] that over 95 % of the injected pyruvate disappears from the blood stream within the first 5 min. Even after taking into consideration the amount which appeared as additional lactate and glucose, the sum of these does not account for more than 10 % of the total quantity injected. The question then arising was whether the remaining 90 % was actually metabolized or had been retained in the muscles! As this question has considerable bearing on the mode of pyruvate disappearance *in vivo* the lactate and pyruvate changes in the muscle and blood after intravenous injection of small doses of pyruvate were studied. The results of these experiments are summarized in Tables II and III.

The blood pyruvate changes confirm the previous observations. Only 2-3 % of the total injected pyruvate is retained in the muscles. Though a definite increase of the lactate content in the blood occurs, the lactate in the muscles may or may not be raised. If ± 6 mg. per 100 g. are allowed as experimental error then 30 % of the cases studied show a lactate increase. No alteration of sugar content was detected in the muscles.

In view of the role of phosphate in the glycolysis in mammalian tissues the possible change of the various phosphate fractions (e.g. inorganic phosphate, 7 min. acid-hydrolysable, triosephosphate and phosphopyruvate) in the blood or muscle were investigated in samples from two rabbits. Under the conditions of these experiments there was no appreciable alteration in any of the phosphate fractions.

Wilkins *et al.* [1938] and Flock *et al.* [1938] have failed to obtain evidence of lactic acid formation when pyruvate is incubated with blood *in vitro*. Our own preliminary experiments of the same type confirm the above observations. However, in two experiments the defibrinated and fluorized blood from normal rats was able to form lactic acid *in vitro* when hexosediphosphate and adenylic acid as well as the pyruvate were provided for the reaction. 0.33 and 0.47 mg. lactic acid can be produced from 1 ml. blood after 2 hr. at 37°. The reaction takes place with coupled esterification of inorganic phosphate in the same manner as found in muscle extract [Needham & Pillai, 1937] and has already been described for blood by Dische [1934]. This observation suggests that at least one of the paths of pyruvate removal involves the oxidation of triosephosphate by pyruvate. It is intended to make later a comparative study of this system in normal and deficiency states.

The observation of an increased lactate and glucose content in the blood of animals after intravenous injection of large doses of pyruvic acid has been reported before [Meyer, 1912; Dakin & Janney, 1913]. These authors concluded that if conditions do not favour the initial reduction to lactic acid no sugar is formed. Recently Margaria & Ponzio [1937], Simola [1937] and Flock *et al.* [1938] also observed that pyruvate injection was followed by an increase of lactate content of the blood. However, the last-mentioned authors did not find an increase of blood glucose after giving continuous injection of pyruvate to dogs. They claim to have obtained a decrease of blood inorganic phosphate in their dogs. On the other hand Kamiya [1936] concluded that intravenous injection of small doses of pyruvate (0.1-2.5 g.) into normal rabbits gives rise to hypoglycaemia. Nevertheless it may be mentioned that Kamiya's conclusion was based on subsequent estimations of blood sugar on samples obtained not a few minutes but several hours after the injection of pyruvate. Since the rate of pyruvate removal in the rabbit is extremely rapid it appears difficult to ascribe the hypoglycaemia produced 2-4 hr. later to the direct effect of pyruvate.

The finding that the injection of pyruvate is followed by little detectable change in the muscle together with the definite increase of lactate in the blood (involving an intermediate stage which can be estimated as B.B.S. or non-glucose reducing substances followed by an increase of glucose) appears to suggest that some other organ, presumably the liver, is concerned with its removal. It has been shown by Meyer [1912] that the fasting rabbit can utilize injected pyruvate to build up glycogen in the liver. The phenomenon of a slight increase of pyruvate accompanied by a much larger increase of lactate in the blood after injection of pyruvate makes it reasonable to suppose that the ability to rebuild carbohydrate from lactate and pyruvate is one of the factors in the organism limiting the efficiency of pyruvate removal. The well-known decrease of glycogen content in the tissues, the similar small increase of pyruvate accompanied by a much larger increase of lactate in the blood and the delayed removal of these after exercise in avitaminous animals appear to indicate that vitamin B₁ may play an important role in the anabolism as well as the catabolism of glycogen.

SUMMARY

1. Estimations of carbohydrate changes in the blood of normal rabbits immediately after intravenous injection of pyruvate suggest that pyruvate injection gives rise to a sudden increase of non-glucose reducing substance and also lactate which afterwards give rise to glucose.

2. Only 2–3 % of the total injected pyruvate can be found in the muscles 8 min. after the injection. There is little or no change in muscle lactate or glucose.

3. When small amounts of pyruvate were injected no appreciable alterations in the phosphate partition were detected in the blood or the muscles.

4. Lactic acid can be formed *in vitro* by incubating blood with pyruvic acid if hexosediphosphate and adenylic acid are also provided. This suggests that pyruvate removal *in vivo* may be closely bound with this important energy-yielding oxido-reduction which is undoubtedly concerned with the regeneration of carbohydrate.

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CXC. DO THE POTASSIUM IONS INSIDE THE MUSCLE CELLS AND BLOOD CORPUSCLES EXCHANGE WITH THOSE PRESENT IN THE PLASMA?

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IN order to find out if and to what extent the potassium ions within the muscle cells and blood corpuscles exchange with those present in the plasma we have administered labelled potassium as potassium chloride (^{42}KCl) to rabbits and frogs and followed its path in the body.

EXPERIMENTAL PROCEDURE

Metallic K was bombarded by deuterium ions of 5.5 million volts energy supplied by a cyclotron. We are most grateful to Prof. Niels Bohr for putting at our disposal the cyclotron and to Dr Jacobsen and Mr Lassen for preparing the active samples. The K was converted into KCl and administered to rabbits by subcutaneous injection or to frogs by injecting it into the lymph sac; it has also been used in experiments *in vitro* [Hahn *et al.* 1939].

The blood samples, after centrifuging to separate the plasma and the corpuscles, and the tissue samples were ashed below 500° , and their activity determined by making use of a Geiger counter. The labelled K (^{42}K) has a half-life of 12.8 hr. No corrections for this decay had to be applied, since all samples were measured relatively to an aliquot of the ^{42}KCl preparation used in the experiments and the results were calculated in percentage of this "standard" preparation. As K has a "natural" radioactivity, due to the presence of ^{40}K in all K samples, we had to apply a correction for the presence of the latter. This was done by measuring the remaining activity of the tissue samples, which was then solely due to the presence of ^{40}K , after the lapse of several days, and subtracting this value from the activity measured at an early date. The maximum extent of the correction to be applied was 10% of the value obtained for the activity of the sample at an early date and amounted in many cases to only 1% or less. The corrected activity of the sample is a measure of its labelled K content.

Experiments on frogs

To *Rana esculenta* var. *red.*, weighing 60, 65 and 58 g. respectively, kept at 22° , 0.7 ml. of a solution containing 3.7 mg. labelled K as KCl was injected. 1 hr. after the start of the experiment one leg was removed and a small plasma sample secured; after the lapse of 24 hr. the animal was killed. The comparison of the activities of the gastrocnemius of the left and right legs, removed after 1 and 24 hr. respectively, is seen in the tables below, which contain also data on the activity of the plasma, the femur epiphysis and diaphysis. The values are expressed relatively to the activity of plasma of the same fresh weight secured 1 hr. after the start of the experiment.

To facilitate the removal of the extracellular labelled K by the Ringer solution several incisions were made in the muscle during the last-mentioned experiment. By this procedure possibly some of the cell walls were destroyed, and a part of the labelled K content of the Ringer solution is of cellular origin.

As seen from the figures below, 1 g. muscle tissue contains more labelled K than 1 g. plasma and, therefore, the labelled K present in the muscle tissue cannot be interpreted as being located in the extracellular volume since this amounts to only about 12 % of the total muscle volume [Fenn & Cobb, 1935; Manery & Hastings, 1939]. At first sight, this result seems to prove the exchangeability of the cellular K. This is not the case, for 1 g. muscle contains about 30 times more K than 1 g. plasma [Fenn, 1936], and, therefore, in the case of an easy exchangeability we should expect 1 g. muscle to contain 30 times as much ^{42}K

Table I

Frog I. Relative activities of fresh samples of equal weight

Sample	Activity after 1 hr. left leg	Activity after 24 hr. right leg
Plasma	100	95
Gastrocnemius	135	146
Epiphysis	152	161
Diaphysis	63	138

Urine secreted during the experiment contains 4.2 % of the labelled K injected. Haematocrite value 21.6.

Table II

Frog II

Sample	Activity after 1 hr. left leg	Activity after 24 hr. right leg
Plasma	100	124
Gastrocnemius	116	148
Epiphysis	135	179
Diaphysis	70	118

Urine contains 4 % of the labelled K injected.

Table III

Frog III. In this experiment the gastrocnemius was, before ashing, immersed into 15 ml. Ringer solution for 2 min.

Sample	Activity after 1 hr. left leg	Activity after 24 hr. right leg
Plasma	100	99
Muscle after immersion	93	113
15 ml. Ringer solution	32	51

as 1 g. plasma. We find, however, the ratio to be only 1.25 in the 1 hr. experiment, and 1.34 in that of 24 hr. duration. Thus about 96 % of the cellular K content does not take part in an exchange process in the course of 1 hr. More significant than the above figure itself is the fact that, when the time of the experiment is increased from 1 to 24 hr., the above figure decreases only to 95.7 %. We thus find an uptake of some labelled K by the muscle tissue: this cannot be interpreted as due to the invasion of the extracellular liquid by labelled K, since the labelled K involved is about 10 times larger than can be taken up by the

extracellular liquid, though only about $1/22$ of the amount which should have been taken up in the course of the experiment had the K ions incorporated into the cells come into exchange equilibrium with those present in the plasma in the course of the experiment.

One could argue that, though the exchange equilibrium stage is not reached after the lapse of 1 hr., it may be obtained after the lapse of several hours. But, as seen above, in the course of 24 hr. only a very modest increase is reached, showing that we are not faced with a progressive process of reasonable speed, but mainly with a rapid influx of labelled K into the muscle tissue in the early stages of the experiment. Possibly only a fraction of the cell K migrates easily through the cell walls or only certain cells present in muscle tissue are easily permeable by K ions. The result of the above experiment shows clearly, however, that the greater part of the cellular K is certainly not exchangeable under the conditions which prevailed in our experiments. The sudden influx mentioned above could also be explained by assuming that an active secretion of K into the muscle cells is engendered by the increase (amounting to a maximum of 50%) in the KCl content of the plasma. If this were the correct explanation, the sudden influx of ^{42}K into the muscle tissue should cease if an increase in the K content of the plasma were avoided by injecting a negligible quantity of labelled K. We intend to carry out this experiment, though the results of experiments with rabbits, given below, in which a K content of the plasma was almost normal throughout the experiment, were very similar to those obtained with frogs.

Experiments on rabbits

The result of an experiment on a rabbit weighing 2.1 kg. is seen in Table IV.

Table IV. *Labelled K content of the organs of a rabbit 24 hr. after subcutaneous injection of 15.5 mg. labelled K as chloride**

Time	Organ	Relative labelled K content
16 min.	Plasma	69
35 "	"	83
61 "	"	93
93 "	"	110
3.0 hr.	Plasma	103
15.5 "	"	103
19.5 "	"	106
24.5 "	"	100
24.5 hr.	Corpuscles	65
24.5 "	Muscles (gastrocnemius)	145
24.5 "	Liver	141
24.5 "	Kidneys	162
24.5 "	Brain	96
24.5 "	Tibia epiphysis	107
24.5 "	Tibia diaphysis	65
24.5 "	Marrow	79
24.5 "	Spleen	110

The ratio found for the labelled K content of 1 g. fresh muscle tissue and of 1 g. plasma after the lapse of 24 hr. is found to be 1.45, while a similar figure (1.35) was obtained in the experiments on frogs.

* The rate of resorption of labelled K in human subjects was studied by Hamilton [1938], in rats by Greenberg *et al.* [1938].

Taking 18 mg. per 100 ml. as the K content of the plasma and 450 as that of the muscle cells, on the assumption of a free exchange of the K ions between cell tissue and plasma, an individual K ion should have $\frac{450}{18} = 25$ times greater chance of being located in the cells than in the plasma of the same weight. Thus we should find about 25 times more ^{42}K in 1 g. muscle tissue than in 1 g. plasma.

It is of interest to remark that a progressive influx of ^{42}K on a larger scale into the muscle cells in the course of 24 hr. is incompatible with the comparatively constant ^{42}K level of the plasma recorded in Table IV. The K content of the total extracellular fluid including plasma of a rabbit amounts to only about 1/30 of the total K content of the intracellular fluid of the muscles and therefore an influx of ^{42}K into the muscle cells would soon rob the plasma of most of its ^{42}K content.

Apparent extracellular volume in experiments with K

Numerous investigations have been carried out by different workers on the change of the K content of muscle tissue during muscular action, recovery tremor etc. [Fenn, 1936]. The conditions prevailing in these experiments were different from those in ours. It is however of interest to compare the results obtained by Bourdillon [1937], and Winkler & Smith [1938], respectively, with those obtained in our experiments. These authors administered K salts by mouth or by intravenous injection to resting human subjects and animals respectively, and determined the apparent extracellular volume from the increase in concentration of the K content of the serum water. The volume of the fluid of the body through which any substance is distributed after injection may be calculated by the following formula.

$$\text{Apparent volume of distribution} = \frac{\text{amount injected minus amount excreted}}{\text{increase in concentration in serum water}}.$$

When calculating the apparent volume of distribution of Cl or Na ions, for example, one arrives at the result that water amounting to about 27 % of the body weight takes part in the dilution of the substances introduced into the plasma, the diluting liquid being considered to be solely extracellular. When carrying out such experiments with K salts, Bourdillon, and also Winkler & Smith, arrived at a value 2 to 3 times as high for the apparent volume of distribution, and this result was interpreted by them as due to the influx of a part of the K into the cells of the body. From their figures it follows that the cell water present in the tissues took up about twice as much K as the extracellular water. The ratio of the K content of the cell water and the extracellular water of the muscles amounts, however, to more than 100. Should the muscle cells be permeable to K ions, the K administered should have been distributed in a ratio larger than 100 : 1 between the cell water and the extracellular water of the muscles. Winkler & Smith determined the volume of K distribution at different times after the start of the experiment. After the lapse of an hour or two no further increase was found, such as we should expect in the case of progressive intrusion of the excess of K into the cells. In fact, in many cases a decrease with time after the lapse of about $1\frac{1}{2}$ hr. was observed by them.

While in the above experiments the large values obtained for the volume of K distribution were interpreted as due to the influx into the cells, it is quite possible that the mineral constituents of the bone tissue take up a part of the excess K introduced into the circulation. It is quite conceivable that in part the circulation is getting rid of its excess K by incorporating K ions into the bones

in place of some of the Ca or Na atoms of the latter. We are led to this possibility by the results obtained when investigating the ^{42}K uptake by the bones of frogs and rabbits.

Uptake of labelled K by the bones

As seen in Tables I, II and IV, 1 g. of fresh femur epiphysis takes up more labelled K than found in the plasma of equal weight. The water content of the epiphysis amounts to about 25 % of its weight. This water can be considered to be mostly of extracellular origin. Therefore we can assume about 1/4 of the ^{42}K found in the epiphysis to be extracellular K, while the rest will presumably be mainly that which is incorporated into mineral constituents of the bone.¹ As we must suppose that other individual K ions present simultaneously in the plasma will show the same behaviour as ^{42}K , we can conclude that in the above experiments on frogs 1 g. fresh epiphysis tissue took up about 0.1 mg. of the K ions which were located in the plasma after the start of the experiment. These K ions presumably exchanged with those present in the bone. Such an exchange process presumably goes on in such a way that some of the K ions of the bone are released into the lymph and an equal number during the same time in the opposite direction. If we disturb this equilibrium by introducing into the plasma a K excess, it is probable that more K ions than before will enter into the bones during a given time, their uptake being compensated by a release of other cations present in the bone.

The diaphysis takes up markedly less labelled K than the epiphysis. Similar results were obtained by us in numerous cases when investigating the labelled phosphate uptake by bones of diverse animals [Hevesy *et al.* 1937], and also by Dolls *et al.* [1939]. The difference is probably due to the less effective lymph circulation in the diaphysis. An exchange or uptake of ions into the mineral constituents of the bone can only take place if these constituents are in contact with the lymph from which the uptake takes place. The diaphysis contains less water and correspondingly less K in the water phase, which can, however, account for only a minor part of the difference in the uptake of labelled K.

Rate of penetration of the phosphate ions into the muscle cells

It is of interest to compare the rate of penetration of K ions with that of phosphate ions into the tissue cells. When working with labelled phosphate, due regard must be taken of the rapid exchange of the plasma phosphate with that of the bones and other tissues, which leads to a rapid decrease of the labelled phosphate content of the plasma. To avoid the latter we inject the labelled Na phosphate drop by drop subcutaneously into rabbits during the experiment. After the lapse of 4 hr. we find that 1 g. fresh gastrocnemius tissue contains 0.6 times as much labelled P as does 1 g. plasma. The labelled P content of the muscle tissue is partly located in the extracellular space. Making use of the data discussed on p. 1555, we reach the result that the extracellular P content of 1 g. fresh gastrocnemius tissue amounts to 1/12 of that of an equal weight of plasma. It follows that the cellular labelled phosphate present in 1 g. gastrocnemius tissue amounts to 0.51 times that in 1 g. plasma. As we find the inorganic P content of the plasma of the rabbits to be 4.2 mg. per 100 ml., it follows that in the course of 4 hr. 0.02 mg. P originally located in the plasma migrated into

¹ As the epiphysis contains about the same amount of ^{42}K as muscle tissue of the same weight, and the cellular part of the bone makes up about 1/10 of the bone weight, the bone cells should take up much more ^{42}K than do the muscle cells to account for the non-extracellular ^{42}K present in the epiphysis.

the cells of each g. of the gastrocnemius, while an equal amount of non-labelled P migrated in the opposite direction.

The rate of penetration of the phosphate ions into the muscle cells depends not only upon the properties of the cell wall but also upon the rate at which the labelled inorganic P is incorporated into the organic compounds inside the cell, and thus is prevented, for the time being, from diffusing back into the plasma. Simultaneously with the formation of these new labelled organic molecules the decomposition of an equal number of "old" organic molecules takes place. This produces non-labelled phosphate ions, which "dilute" the active inorganic P content of the cells. When evaluating the number of labelled P atoms which pass the cell wall during the experiment we must consider the labelled inorganic and organic P molecules present in the cells as well. We then arrive at the result that the number of phosphate ions migrating from the plasma into the cells during the course of the experiment is equivalent to about 1/80 of the total acid-soluble P present in the cells.

Exchangeability of the K of the corpuscles

As seen in Table IV, in blood samples removed from a rabbit 24.5 hr. after the start of an experiment the labelled K is distributed between equal weights of plasma and corpuscles in the ratio 100 : 65. We also determined this ratio after the lapse of 2 hr., and found it to be 100 : 20. An appreciable part of ^{42}K ions, and thus a corresponding part of all the K ions originally present in the plasma, was found in the corpuscles. We must, however, remember that the corpuscles contain about 20 times as much K as does the same weight of plasma. In case of a complete exchangeability of the K of the corpuscles we should have found 20 times as much ^{42}K in the corpuscles as in the plasma or an effect about 30 times larger than found in the above-mentioned experiment after the lapse of 1 day.

Experiments in vitro

In experiments *in vitro* rabbit's blood was shaken, after addition of labelled KCl, in a $\text{CO}_2\text{-O}_2$ atmosphere at 37° for 2 hr. After centrifuging and rapidly washing the corpuscles twice with non-active plasma of another rabbit (2 g. plasma for 1 g. corpuscles in each case), we found the ratio of ^{42}K in equal weights plasma and corpuscles to be 100 : 35. Adding the ^{42}K found in the washing plasma to the amount found in the corpuscles, the above ratio becomes 100 : 38.

In other experiments labelled KCl was added to the blood sample and, after shaking for 1 hr., the corpuscles were separated. The latter were not quickly washed with non-active plasma, as described above, but shaken with non-active plasma for half an hour at 37° . This operation was repeated twice more, the corpuscles being separated each time by centrifuging. The results obtained are seen in Table V.

Table V. *Distribution of labelled K between plasma and corpuscles in blood samples (ratio of volume of plasma and corpuscles 2 : 1)*

	Corpuscles before treatment with non- active plasma	First washing plasma	Second washing plasma	Third washing plasma	Corpuscles after treatment with non- active plasma
Plasma					
88%	12%	3.1%	1.2%	0.47%	7.2%

The ratio of the ^{42}K content of equal weight of plasma and untreated corpuscles equals 100:21. It is seen from the above figures that, when corpuscles into which ^{42}K had been previously incorporated during an experiment lasting 1 hr. were shaken for $1\frac{1}{2}$ hr. with non-active plasma again, only 40% of the ^{42}K taken up by the corpuscles phase could be removed. This suggests that besides adsorption of ^{42}K on the surface of the corpuscles and penetration into the wall of the latter a leak into the interior of the corpuscles took place as well. The corpuscles present in the blood are a mixture of individual corpuscles differing in age and resistance and it is possible that only particular ones exhibit such a leakage. Henriques & Ørskov [1939] have shown in a recent paper, which is of interest in this connexion, that the blood of animals in which strong anaemia was produced, and which thus contains a large percentage of young corpuscles, contains corpuscles with a very high K content. Experiments on such animals may throw light on the point raised above.

From the above considerations it follows that the permeability of an average corpuscle of a fully grown rabbit to K ions is certainly very minute, or, more correctly, the bulk of the K ions present in such corpuscles is not replaced in the lifetime of the latter.

Experiments with labelled Na

We also subjected rabbits to subcutaneous injections with labelled Na as $^{24}\text{NaCl}$. In blood samples taken after the lapse of about 1 day, we found, on an average, that ^{24}Na was distributed between equal weights of plasma and corpuscles in the ratio of 100:6 when the corpuscles were washed twice with non-active plasma and recovered each time by centrifuging. The ratio was 100:14 when the activity of the unwashed thoroughly centrifuged corpuscles was compared with the activity of the plasma of the same weight. An equipartition of ^{24}Na between equal weights of corpuscles and plasma would correspond to a ratio of about 100:25.

A large amount of ^{24}Na was found in these experiments in the plasma employed in washing the active corpuscles after their separation being in some cases even larger than the remaining ^{24}Na content in the corpuscles. In view of the large Na content of the plasma and the comparatively low Na content of the corpuscles of rabbit's blood, Na is less suitable than K for studying the interpenetration of alkaline ions. The results obtained suffice, however, to show the great difference in the behaviours of Na and K in the corpuscles.

Distribution of Na in the organs

As is to be expected, 1 g. of muscle tissue takes up much less labelled Na than K, for the Na uptake is known to be confined to a very large extent to the extracellular fluid of the muscle tissue. From the extent to which labelled Na injected into the veins of a rabbit became diluted Griffiths & Maegraith [1939] calculated that the volume of fluid in which the ^{24}Na was dissolved must have been about 840 ml., amounting to 36% of the body weight in water. In other animals they found values between 30 and 35%. Owing to the high activities at our disposal we could compare the activity of 1 g. plasma with that of 1 g. muscle tissue, which is the most direct method of determining the extracellular volume of the tissue in question. We find the ratio of the ^{24}Na content of 1 g. plasma to that of 1 g. gastrocnemius to be 100:8.5. Manery & Hastings [1939] found an almost identical figure, 8.6, for the ratio of the Na content of 1 g. plasma and of 1 g. fresh gastrocnemius tissue of a fully grown rabbit. Our

experiment leads thus to the same value for the volume of the extracellular space of the gastrocnemius muscle as stated by them, namely 11, expressed as g. per 100 g. blood-free, fat-free tissue. We determined also the extracellular space of the total body, as we will discuss below.

The distribution of ^{24}Na between 1 g. plasma and 1 g. fresh tissue of different organs, 67 hr. after subcutaneous injection of 23 mg. labelled Na to a rabbit weighing 2.2 kg., is seen in Table VI.

Table VI. *Distribution of labelled Na between plasma and fresh tissue of equal weight*

Organ	Relative ^{24}Na content
Plasma	100
Gastrocnemius	8.5
Heart	30.4
Liver	33.7
Kidneys	53.4
Spleen	29.6
Lungs	34.5
Marrow	35.3
Brain	32.0
Tibia epiphysis	59.1
Tibia diaphysis	50.9

In these experiments of long duration (67 hr.), a part of the ^{24}Na found in some of the tissues is possibly not of extracellular origin. This is certainly the case for the bones. As only 1/4 of the latter is water, the extracellular ^{24}Na content of the bone tissue cannot be more than 1/4 of that of an equal weight of plasma. However, about twice this much was found. Since the non-labelled Na ions also present in the plasma must show the same behaviour as ^{24}Na , we can conclude that, of the 3 mg. Na present in 1 g. plasma, about 0.8 mg. exchanged in the course of 67 hr. with Na atoms present in 1 g. epiphysis at the start of the experiment. From entirely different considerations Harrison [1937] (cf. also Harrison *et al.* [1936]) came to the conclusion that 42 % of the total bone Na is not extracellular, which compares well with our results.

To determine the variation of the ^{24}Na content of the plasma with time we carried out the following experiment. 23 mg. ^{24}Na , as NaCl, were injected into rabbit A. After the lapse of 3 days 35 ml. of blood were removed from rabbit B, the corpuscles were separated and mixed with 29.6 ml. of plasma from rabbit A and the blood thus obtained was introduced into rabbit B, the injection requiring 2 min. The ^{24}Na content of the 29.6 ml. plasma injected into rabbit B was found, after the lapse of 3 min. (excluding the time of injection), to have been brought

Table VII. *Change of the labelled Na content of the plasma with time, after intravenous injection*

Time	% of ^{24}Na injected present in 1 g. plasma	% of ^{24}Na injected present in total plasma (assumed to be 80 ml.)
0 min.	—	100 (extrapolated)
2 "	0.164	13.1
5 "	0.158	12.7
15 "	0.155	12.4
33 "	0.150	12.0
56 "	0.110	8.8
203 "	0.104	8.3
47 hr.	0.069	5.5

down, by dilution by the body fluids, to $1/20.6$ of its initial value. The volume of the diluting fluid must have amounted to 610 ml. or 28 % of the body weight. In calculating the above figure no regard was taken of the possible uptake of some ^{24}Na by the mineral constituents of the bones. This figure compares well with that found by entirely different methods for the value for the extracellular volume of the total body of a rabbit. The rapid fall in the ^{24}Na content of the plasma witnessed immediately after the start of the experiments is followed by a moderate decrease with time as seen in Table VII. This decrease is possibly due to some extent besides excretion to penetration of ^{24}Na into the non-extracellular part of the tissues.

SUMMARY

Labelled K as ^{42}KCl was administered to rabbits and frogs and its penetration into the corpuscles, the muscles and other organs was investigated.

It was found that, after the lapse of 1 day, corpuscles contain about 60 % as much ^{42}K as the same weight of plasma. Thus some uptake of ^{42}K by the corpuscles takes place. The amount of ^{42}K found in the corpuscles, however, is about 30 times less than is to be expected in the case of an equipartition of ^{42}K between the K ions of the corpuscles and those of the plasma. The bulk of the K ions present in the corpuscles of a fully grown rabbit is thus not replaced in the lifetime of the corpuscles.

The gastrocnemius of frogs contains, after the lapse of 1 hr., 1.25 times, and after the lapse of 24 hr., 1.35 times as much ^{42}K as does the same amount of blood plasma at the same time. Its labelled K content is thus about 10 times larger than expected on the assumption that all the ^{42}K taken up by the muscles is present in the extracellular space. The amount of ^{42}K found, however, is about 20 times less than expected assuming an exchangeability of all K present in the muscle cells. A similar result was obtained with rabbits.

The ^{42}K content of the skeleton was found to be about double that expected if all ^{42}K is confined to the extracellular space.

The rate of penetration of phosphate ions into the muscle cells is discussed.

After the lapse of a day about $1/2$ of the Na in the corpuscles was replaced by ^{24}Na .

Gastrocnemius tissue contained 8.5 % of the ^{24}Na content of the same weight of plasma, the corresponding figures for the tissues of all other organs were found to be much larger.

The extracellular space of the total rabbit was calculated to be 28 % of the body weight.

Note added 28 September 1939. In a recent paper Joseph *et al.* [1939] record a low permeability of rat's muscle to labelled K. A remarkably high permeability of the corpuscles of the dog to labelled sodium was found by Cohn & Cohn [1939].

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CXCI. METABOLISM OF SULPHUR

VIII. OXIDATION OF THE SULPHUR-CONTAINING AMINO-ACIDS BY ENZYMES FROM THE LIVER OF THE ALBINO RAT¹

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THE paths of oxidation of the sulphur-containing amino-acids in the animal body have been deduced largely from quantitative and qualitative studies of the end-products excreted under various metabolic conditions. An attempt has been made in this investigation to extract the enzymes concerned in these transformations and to study some of the conditions under which they operate. The present paper is concerned, for the most part, with the oxidation of cystine and cysteine and of some of their partially oxidized derivatives.

Methods

Cysteine and cystine were determined by the method of Shinohara & Padis [1935]. Protein-free filtrates were prepared by adding 1.5 ml. of the solution to be tested to 13.5 ml. of sodium tungstate-sulphuric acid mixture in a 15 ml. centrifuge tube.

Protein precipitant (modified from Folin and Svedberg, for preparation of protein-free filtrates): 15 g. of sodium tungstate are dissolved in about 200 ml. water, 30 ml. $\frac{2}{3}$ *N* H_2SO_4 added and the volume made up to 250 ml.

By this method, cytochrome *c*, which interferes in the determination of cysteine with phosphotungstic acid, is precipitated along with the tissue proteins. When the precipitate has begun to flocculate, the tubes are centrifuged and 5 ml. of the supernatant fluid are transferred to each of two 25 ml. volumetric flasks for the determination of SH and SS groups. For the former, 6.5 ml. of sodium acetate-acetic acid buffer at pH 5.2 are added, followed by 2 ml. of the phosphotungstic acid uric acid reagent. For determination of disulphide, 2 ml. of 1 *M* sodium sulphite are added just before the phosphotungstic acid reagent. For the standard, 4 ml. of 0.002 *M* cysteine, 6.5 ml. of buffer and 2 ml. of reagent are prepared in a third 25 ml. volumetric flask. The contents of the flasks are then made up to 25 ml., and colorimetric readings are taken at the end of 20 min.

Sulphate was determined by three different methods developed during the course of the investigation. Repeated checks indicated agreement in the values obtained. The methods were as follows.

(a) Pirie's [1934] modification of Cuthbertson & Tompsett's [1931] method.

(b) A slight modification of Denis & Reed's [1926-7] procedure for the nephelometric determination of sulphate in blood serum. 8 ml. of the solution con-

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taining the tissue slices or tissue extract are added to 2 ml. of 20% sulphate-free trichloroacetic acid, and centrifuged after about 20 min. To 2 ml. of the filtrate are added 5 ml. water and 1 ml. of 5% gelatin. Finally, 2 ml. 1% BaCl_2 are added with continuous mixing with a fine glass rod. A blank is prepared substituting water for the BaCl_2 and a series of $(\text{NH}_4)_2\text{SO}_4$ standards containing 12, 24 and 36 μg . S respectively. The amount of sulphate present as sulphur is read, after deducting the reading of the blank, from a curve constructed by comparing graded amounts of the standard sulphate solution with each of the several standards.

(c) An adaptation of the nephelometric method for use with the photometer [Medes & Stavers, 1939].

Experiments with tissue slices

Pirie [1934] first succeeded in demonstrating that slices of liver and kidney of the rat are capable of oxidizing the sulphur of cystine, cysteine, methionine and glutathione to inorganic sulphate. He states that finely ground tissue is inactive. Pirie concerned himself only with sulphate formation. Experiments conducted similarly were carried out in this investigation to obtain a survey of other reactions which may be occurring in liver tissue. Albino rats were also employed, the entire procedure being carried out as described by Pirie, using the organ-slice method of Warburg. A constant temperature bath at $38.5^\circ \pm 0.2$ was used. All experiments, unless otherwise stated, were continued for a 2 hr. period with a 95% O_2 -5% CO_2 mixture bubbling continuously through the solutions. The carbonate buffer of Krebs with substitution of magnesium chloride for magnesium sulphate, as suggested by Pirie, was employed for the most part. In later experiments, especially with some of the extracted enzymes, 0.2 *M* KH_2PO_4 -NaOH buffer at pH 7.5 was substituted. In this case pure O_2 was used for aeration in place of the O_2 - CO_2 mixture. Sulphate formations by different tissues are recorded in Table I.

The results agree in general with those reported by Pirie. Kidney and liver oxidize the compounds most readily; blood, spleen and lung tissue are inactive, while cardiac and heart muscles possess a low degree of activity. Duodenum rapidly gives rise to sulphate; even without added cysteine, its output was high as compared with those of the other tissues, except kidney and liver. In general, sulphate formation from cystine was somewhat less in a given period than from cysteine; cystine disulphoxide yielded about the same amount in the 2-hr. period as did cystine, while the most rapid conversion was from cysteinesulphinic acid. The slow production of sulphate from cystine as compared with that from cysteine tends to confirm the finding of Pirie that cystine does not constitute the immediate source, but reduction to cysteine must take place. The same conclusion would apply to cystine disulphoxide. The yield of sulphate from cysteine, as seen from Table I, varied, under the conditions of these experiments, being around 3% of the added sulphur. When phosphate buffer was substituted for carbonate buffer, sulphate formation from cysteinesulphinic acid was undiminished, whereas no sulphate was produced from cysteine.

A second reaction also occurs. The greater portion of the cysteine is converted into cystine under these conditions. In Table II, the yields of cystine in a series of experiments are recorded; 53-89% appeared as cystine at the close of the 2-hr. periods. This oxidation to cystine takes place with equal facility in phosphate and carbonate buffers.

It may be seen also that a third reaction is taking place. The recovery of cysteine plus cystine (final column) varies from 65 to 89% of the initial cysteine.

Table I. *Conversion of organic S into inorganic SO₄ in carbonate buffer at pH 7.4*

10 mg. (8.26 m. equiv.) of the compound to be tested in 10 ml. of the buffer were aerated throughout by a 95% O₂-5% CO₂ gas mixture. Time, 2 hr. Temp. 38° ± 0.2.

Compound	Tissue slices	Sample no.	Tissue av. wt. mg.	Compound used m.M. × 10 ³	SO ₄ formed as S m.M. × 10 ³	SO ₄ formed as S μg.
<i>l</i> -Cysteine	Liver	26	56.97	8.26	3.49	79.90
"	Kidney	12	36.61	8.26	2.02	64.94
"	Blood	4	61.07	8.26	0.19	6.37
"	Spleen	5	26.84	8.26	0.38	12.30
"	Duodenum	1	14.90	8.26	1.67	53.80
"	Lung	4	22.32	8.26	0.48	15.67
"	Heart muscle	6	47.66	8.26	0.91	29.31
"	Muscle (striped)	4	78.92	8.26	1.24	40.05
<i>l</i> -Cystine	Liver	8	58.73	4.13	1.83	58.7
"	Kidney	7	46.04	4.13	1.15	37.1
"	Muscle	4	79.70	4.13	1.07	34.4
"	Heart muscle	3	57.53	4.13	1.25	40.1
<i>l</i> -Cystine disulphoxide	Liver	8	59.48	4.13	1.85	59.5
"	Kidney	7	45.62	4.13	1.42	45.6
"	Muscle	4	78.20	4.13	1.19	38.2
"	Heart muscle	3	59.30	4.13	1.22	39.3
"	Spleen	1	31.70	4.13	0.55	17.7
<i>l</i> -Cysteinesulphinic acid	Liver	8	58.42	8.26	2.30	73.9
"	Kidney	7	47.50	8.26	3.01	96.6
"	Muscle	4	77.85	8.26	2.32	74.4
"	Heart muscle	3	57.23	8.26	1.66	53.5
"	Spleen	1	29.00	8.26	0.38	12.5
Control	Liver	15	52.54	—	0.47	15.1
"	Kidney	8	30.72	—	0.51	16.6
"	Blood	4	61.67	—	0.22	7.2
"	Spleen	5	28.47	—	0.38	12.3
"	Duodenum	1	14.80	—	1.38	44.4
"	Lung	4	22.62	—	0.42	13.7
"	Heart muscle	3	34.43	—	0.24	8.0
"	Muscle	2	76.20	—	0.33	10.7

Table II. *Cystine formation from cysteine in the presence of liver slices in carbonate and phosphate buffers at pH 7.6. Temp. 38.5° ± 0.2*

Buffer	Cysteine mg.	C	Time min.	SH mg.	SS mg.	Total mg.	Time min.	SH mg.	SS mg.	Total mg.
Carbonate	10	+	60	2.25	4.74	6.99	120	1.71	5.32	6.45
Carbonate	10	+	60	6.40	3.86	10.26	120	2.12	6.14	8.26
Carbonate	10	+	60	5.03	4.24	9.45	120	0	8.88	8.88
Phosphate	10	—	60	9.1	0.7	9.8	120	2.4	6.0	8.40
Phosphate	10	+	60	3.04	6.02	9.06	120	0.51	7.38	7.89

Since only about 3% of the sulphur appears as inorganic sulphate, some reaction is taking place by which neither cystine nor inorganic sulphate is produced. In the present investigation attempts were made to study, through the use of liver extracts, these three reactions as well as sulphate formation from cysteine-sulphinic acid.

Studies with liver extracts

As a preliminary to the study of the several reactions, a general survey was made by carrying out a series of experiments substituting liver brei for liver slices.

Preparation of liver brei. The rat was killed by a blow on the head and the liver immediately perfused with physiological salt solution, first by way of the arterial system from the heart, then by way of the venous system through the hepatic portal and the hepatic veins. When the deep reddish colour was superseded by a uniform pale greyish cast, the liver was removed and ground in phosphate or carbonate buffer either in a Latapie mincer or in a mortar with sand. After standing with occasional stirring for 30 min., it was centrifuged and the supernatant liquid filtered through a Jena glass Büchner funnel of medium porosity (3G 3). Repeated examinations failed to demonstrate any intact liver cells in the filtrate. 4 ml. of the liver extract were used in each tube, usually 0.5 ml. of cytochrome *c*, 1 ml. of water, buffer to make a total volume of 10 ml. and 10 mg. of cysteine or its sulphur equivalent. At this concentration, as observed by Pirie, cystine formed from cysteine just fails to precipitate out, and hence aliquot parts may be removed at intervals for determination of thiol and disulphide.

Table III. *Oxidation of cysteine to cystine in the presence of (a) crude liver brei, filtered through a sintered glass filter, and (b) oxidase from brei purified similarly to beef-heart cytochrome oxidase*

Brei no.	Treatment of brei	Cytochrome <i>c</i>	No. of exp. av.	45 min.		90 min.		Total SH + SS	
				SH	SS	SH	SS	45 min.	90 min.
				mg.	mg.	mg.	mg.	total mg.	total mg.
1	Crude	+	8	4.45	3.92	0.39	6.55	8.37	6.94
2	Crude	—	6	5.29	3.15	0.73	5.90	8.44	6.63
3	Crude, heated	+	5	7.39	2.53	5.96	4.14	9.92	10.10
4	Purified	+	8	4.70	5.29	1.11	8.36	9.99	9.47
5	Purified	—	5	7.22	2.43	3.28	6.81	9.65	10.09
6	Purified, heated	+	8	7.39	2.51	3.64	6.26	9.90	9.90

In Table III, the first three items record a typical experiment in which crude liver brei in phosphate buffer was employed. As with slices, the most vigorous reaction was that concerned in the oxidation of cysteine to cystine. When the extract was not heated a loss of cysteine plus cystine occurred, only 66 and 69 % being recovered. When the extract was heated for 5 min. at 80° before addition of the cysteine no loss of cystine plus cysteine was seen, demonstrating that the reaction involving loss is enzymic. Sulphate formation occurred only in carbonate buffer and then to a much reduced degree. It also failed to occur when the extract had been heated. In general, then, the reactions which take place upon substitution of the brei for liver slices, were qualitatively and, except for sulphate formation, quantitatively similar to those in which the tissue itself was used.

(a) *Cytochrome oxidase.* Cytochrome oxidase, formerly designated indophenol oxidase, is well known to catalyse the oxidation of cysteine to cystine. Since this enzyme is apparently present in all tissues, it seemed most probably the enzyme responsible for this oxidation.

Cytochrome oxidase for comparative studies was prepared from beef-heart by the method of Stotz & Hastings [1937] and purified by three precipitations in 0.05*M* acetate buffer at pH 4.5 as described by Stotz *et al.* [1938] with subsequent dialysis for at least 18 hr. in frequent changes of glass-distilled water. Cytochrome *c* was prepared according to the procedure of Keilin & Hartree [1937].

As a preliminary to the study of the enzymic oxidation of cysteine to cystine its oxidation in oxygen-saturated phosphate buffer at pH 7.5 and 38.5° was

followed. In Fig. 1 is plotted a group of such curves (*a* to *g*). When temperature and oxygen saturation were maintained uniformly from the start, rate of disappearance of cysteine was represented by a straight line as in curves *e*, *f* and *g*. Therefore rate of oxidation is independent of concentration. The degree of slope varied widely in different experiments, since traces of impurities greatly influence

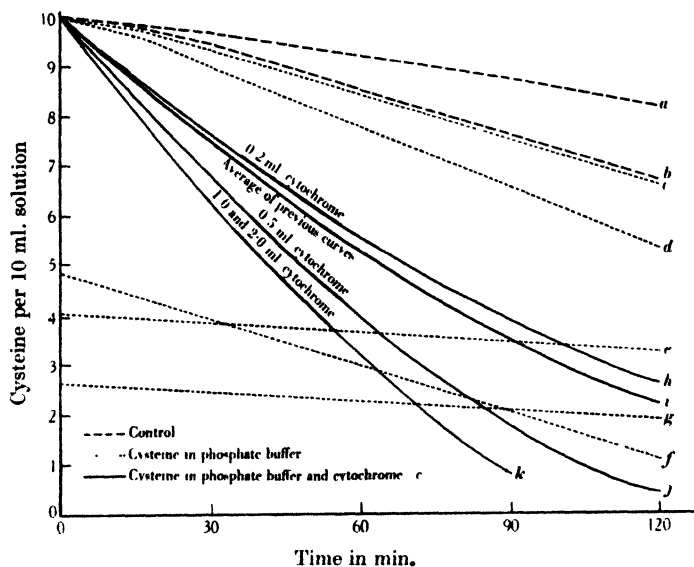


Fig. 1. Rate of oxidation of cysteine to cystine in 0.04 *M* phosphate buffer at pH 7.5 at $38.5^{\circ} \pm 0.2$ in a constant stream of oxygen. Abscissae, time in minutes; ordinates, mg. cysteine per 10 ml. solution. Curves *a* and *b* are controls from previous experiments; *c* to *g* similarly are pure cysteine in phosphate buffer; *h* to *k* contain cytochrome *c* in addition.

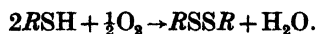
Table IV. Oxidation of cysteine to cystine

48.4 mg. cysteine were introduced into a 100 ml. volumetric flask containing 0.04 *M* phosphate buffer at pH 7.5. The buffer had been saturated previously with oxygen, and the volume was adjusted with additional oxygenated buffer. Oxygen was bubbled through the solution throughout the experiment. Temp. $38.5^{\circ} \pm 0.2$.

Time min.	SH mg.	SS mg.	Total determined mg.
0	48.5	0	48.5
15	44.4	3.9	48.3
30	39.8	8.6	48.4
45	34.2	14.2	48.4
60	29.5	19.1	48.6
90	21.3	27.2	48.5
120	11.5	37.2	48.7
150	2.4	46.0	48.4
180	0	48.7	48.7

the rate, as has been amply brought out by previous investigators. In Table IV is recorded a typical experiment in which 48.3 mg. were dissolved in 0.04 *M* phosphate buffer previously saturated at 38.5° with oxygen. The volume was adjusted to 100 ml. with additional oxygenated buffer and the solution maintained at $38.5^{\circ} \pm 0.2$ in a stream of oxygen. After 3 hr. all the cysteine was

converted into cystine. Recovery of cystine plus cysteine was at all times 100 % of the initial cysteine: hence the reaction conformed to the equation



In another series of experiments, cytochrome *c* was added to ascertain its effect on the rate of oxidation of cysteine and its effect on the type of curve. With cytochrome *c* present, the rate of reaction was always increased. In an experiment conducted simultaneously with that recorded in Table IV, complete oxidation of cysteine to cystine had occurred after 120 min. instead of 160 min. (as read from the curve) in the experiment without cytochrome *c*. The type of curve was also changed (Fig. 1, *h* to *k*), the rate of oxidation no longer being independent of cysteine concentration. Addition of increasing amounts of cytochrome *c* also influenced the rate up to a certain maximum, after which further addition produced no more increase. Recovery of cysteine plus cystine was 100 % of the initial cysteine.

When liver extract was purified by the same procedure as was used in the preparation of cytochrome oxidase, all the initial cysteine was recovered as cysteine plus cystine, as shown by the last three items in Table III. The increased rate upon addition of cytochrome *c* to the system (item 4), above the rate in its absence (item 5), may be taken as further indication that the enzyme is cytochrome oxidase. Further evidence in this direction may be obtained by its behaviour in the presence of cyanide. As shown by Keilin [1929] and later by Stotz *et al.* [1938], cyanide prevents the reoxidation of cytochrome *c* and hence should inhibit the oxidation of cysteine by cytochrome oxidase. Fig. 2 shows an

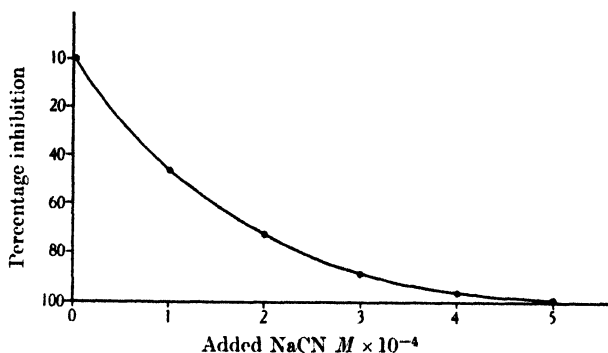


Fig. 2. Inhibition of oxidation of cysteine to cystine by sodium cyanide. The solutions contained 4 ml. purified cytochrome oxidase from rats' liver, 0.5 ml. cytochrome *c*, 1 ml. water and 4.5 ml. phosphate buffer at pH 7.5. Abscissae, added NaCN $M \times 10^{-4}$; ordinates, percentage inhibition of SH to SS.

experiment in which graded amounts of sodium cyanide were added to 10 mg. of cysteine in phosphate buffer in the presence of the purified enzyme and cytochrome *c*. As may be seen, the accelerating effect of cytochrome *c* was more and more inhibited in the presence of increasing amounts of cyanide, until nearly 100 % inhibition was attained. A similar effect was produced by urethane.

It may be concluded then, that the enzyme which is present in the liver of the albino rat and which oxidizes cysteine to cystine is cytochrome oxidase.

This enzyme has no effect on methionine. In one experiment in which cysteine was replaced with 1.25 mg. methionine per ml., 100 % of the methionine was recovered after 2 hr., as determined by Kassell & Brand's [1938] modification of

Baernstein's method.¹ No sulphhydryl was detectable with nitroprusside, and no sulphate with barium chloride. Iodimetric titration indicated that the enzyme does not affect cysteinesulphinic acid either.

(b) *An enzyme oxidizing cysteine to cysteic acid.* In the procedure for extracting and purifying cytochrome oxidase, the enzyme is precipitated from the tissue extract in sodium acetate buffer at pH 5.0. The precipitation is incomplete. In the supernatant liquid containing small amounts of cytochrome oxidase is to be found a second enzyme responsible for the loss of sulphhydryl plus disulphide. In the first experiment recorded in Table V it may be seen that oxidation of cysteine to cystine is occurring, both in the heated and in the unheated specimens—in the latter, with much greater rapidity. In the unheated tube, the loss of sulphhydryl plus disulphide is 3.32 mg. after 1 hr., whereas none has disappeared from the heated tube. After 5 hr. in the unheated tube all the sulphhydryl plus disulphide has disappeared but none from the other. Since at the close of the 2 hr. period 4.74 mg. had been present as disulphide in the unheated tube, disulphide as well as sulphhydryl must be acting as substrate.

To determine if cystine is an intermediate in this oxidation reaction of cysteine or if cysteine is the immediate source, the rates of disappearance of cystine and cysteine were compared in the next two experiments in Table V. Cystine was used as sodium cystinate since this dissolves in the phosphate buffer and at the concentration of about 1 mg. per ml. is approximately at the point of saturation. As may be seen there is a slow conversion of cysteine into cystine, while at the same time a gradual loss of the total amino-acid occurs. The rate of loss is about the same from the cystine solution as from the cysteine. The fourth experiment is similar to Exp. 3 except that crude filtered suspension of liver was employed instead of the partially purified. In this instance loss of cystine plus cysteine is even more rapid in the tube in which cystine acted as substrate. These results suggest either that cystine is the immediate substrate or that here there is some mechanism rapidly reducing cystine to cysteine. If this reduction is enzymic, here is the only indication of enzymic reduction of disulphide to thiol which this investigation has revealed.

The end-product of the reaction appears to be cysteic acid as indicated by iodimetric titration. Amino-nitrogen is unchanged. Since it has been shown by Virtue & Doster-Virtue [1939] that cysteic acid may serve as the precursor of taurine, it seems probable that we are dealing with an enzyme concerned in the production of taurine.

This enzyme appears to be identical with that described by Bergheim & Bergheim [1939], who employed a suspension of liver tissue in phosphate buffer, at pH 6.7. They state that it does not oxidize cystine. As suggested by them, the difference in the behaviour of cystine may be due to the more alkaline reaction at which these experiments were carried out. That there is a difference in behaviour at these two reactions is further demonstrated by the suppression of this reaction by sodium pertitanate at pH 6.7, as shown by Bergheim & Bergheim, although no inhibition occurs at pH 7.5. The final experiment in Table V was carried out in phosphate buffer at pH 6.7. The enzyme had been purified as described above except that phosphate buffer at the lower pH was used throughout. Sodium pertitanate, prepared as described by Bergheim & Bergheim was added. The results show the inhibition of cysteic acid formation.

An attempt was made to determine whether the enzyme could act on cysteinesulphinic acid since the latter compound might be an intermediate in this oxidation of cysteine. Three test tubes were set up, each containing 4 ml. of

¹ I am indebted to Mr Thomas Callan for estimation of methionine.

the partially purified brei, 4 ml. phosphate buffer, 0.5 ml. cytochrome *c*, 1.5 ml. water and 30 mg. cysteinesulphinic acid. From tube *a*, 8 ml. were removed at once for iodimetric titration. Tube *b* was heated in a boiling bath for 2 min., then incubated at 38.5° as a control for tube *c*, for 3 hr. At the close of this period, 8 ml. were removed from tubes *b* and *c* for iodimetric titration. To each of these three 8 ml. portions in 15 ml. centrifuge tubes, 2 ml. of 20 % trichloroacetic acid were added, the solutions centrifuged, and 8 ml. of the supernatant liquid titrated with sodium thiosulphate. The results of two such experiments are given in Table VI. It may be seen that a loss of about 17 % of cysteinesulphinic acid occurred in the presence of the active enzyme (tubes *c*). During a third 3-hr. period 20 % of an equivalent amount of cysteine (23.7 mg.) was oxidized to the cysteic acid stage. The close agreement of these two figures, 17 and 20 % respectively, indicates that cysteinesulphinic acid may be a stage in this oxidation of cysteine.

Table VI. *Recovery of cysteinesulphinic acid*

(*a*) Before, (*b*) after (heated), and (*c*) after (unheated) incubation with purified enzyme for 3 hr. at 38.5 ± 0.2. Each portion finally titrated with thiosulphate contained 0.1254 m.equiv. or 19.2 mg. cysteinesulphinic acid.

	Cysteinesulphinic acid mg.	Recovery %
Exp. 1 <i>a</i> . Before incubation	19.2	—
.. 1 <i>b</i> . After incubation (heated)	18.9	98.3
.. 1 <i>c</i> . After incubation (unheated)	16.0	83.4
Exp. 2 <i>a</i> . Before incubation	19.3	—
.. 2 <i>b</i> . After incubation (heated)	19.1	98.6
.. 2 <i>c</i> . After incubation (unheated)	16.1	83.7

(*c*) *Sulphinic acid oxidase*. The enzyme which oxidizes the sulphur of cysteinesulphinic acid to inorganic sulphate is active in crude brei obtained by grinding liver in either carbonate or phosphate buffer. Its activity is retained after filtration of the brei through a sintered glass funnel with medium size pores which retain all intact cells. When the crude filtered brei is brought to pH 5.0 with an acetate buffer the sulphinic acid oxidase remains in the supernatant liquid above the precipitated cytochrome oxidase and is active even after dialysis. Table VII records a typical experiment. It may be seen that compared with the enzyme which oxidizes the sulphur of cystine to inorganic sulphate, this oxidase is exceedingly active, 1167 µg. S being recovered as sulphate, representing 56 % of the sulphur of the added cysteinesulphinic acid. The loss in purifying was about 75 %.

Table VII

Partial purification of the enzyme from liver of the albino rat, which oxidizes the sulphur of sulphinic acid to inorganic sulphate: (*a*) Crude brei in phosphate buffer. (*b*) The cytochrome oxidase precipitated at pH 5.0 ± 0.2 with acetate buffer, the precipitate made up with phosphate buffer to the same volume as the supernatant liquid and both dialysed against repeated changes of glass-distilled water. All experiments were made in phosphate buffer. Results are expressed in µg. S recovered from initial 10 mg. sulphinic acid.

Exp.	Enzyme	Sulphinic acid (mg.)	Treatment	Sulphate-S µg.
<i>a</i>	Crude	10	—	1167
<i>a</i>	"	10	Heated	0
<i>b</i>	Precipitate	10	—	19
<i>b</i>	"	10	Heated	0
<i>b</i>	Filtrate	10	—	268
<i>b</i>	"	10	Heated	0

(d) *Enzyme oxidizing cystine with the production of inorganic sulphate.* The enzyme which oxidizes the sulphur of cysteine to inorganic sulphate is active in the brei obtained by grinding liver tissue in carbonate buffer and filtering through the Büchner funnel. It is readily adsorbed on permutit either from the carbonate buffer or from a dilute neutral salt solution, but cannot be eluted by any methods attempted. It may be precipitated by MgSO_4 or Na_2SO_4 , but the dialysed precipitate was inactive. While repeated microscopic examinations of the active brei have failed to reveal the presence of intact liver cells, occasional nuclei from disrupted cells and a few blood cells were seen. It is possible that they may carry the active enzymes, as these formed elements were always lacking after first steps of purification when the enzymic activity was lost.

DISCUSSION

Conclusions as to the path of oxidation of cysteine in the liver must be more or less tentative, since the several enzymes are imperfectly separated and may even be contaminated with other enzymes attacking the same substrate at different positions in the molecule. In the production of inorganic sulphate from cysteine there is no evidence of step-wise oxidation, viz. addition of each oxygen atom separately, although the possibility is not excluded. It may be that cysteinesulphinic acid is the end-product of the reaction, and that the oxidation of the latter is carried out by the sulphinic acid oxidase. This possibility is suggested, since otherwise it is difficult to postulate a function for the latter enzyme, which seems to be present in larger amounts than any of the others studied. Cysteine and cystine disulphoxide are definitely excluded as intermediates, since sulphate-production from them is slower than from cysteine. It appears probable, then, that oxidation of the sulphur of cysteine to inorganic sulphate occurs exclusively along the line of the sulphydryl series.

There is somewhat more evidence of a step-wise oxidation of cysteine to cysteic acid. Cystine seems to be oxidized with about equal facility, leaving some doubt as to which is the immediate substrate. If cystine must be reduced to cysteine before being oxidized along the thiol series, the reduction would seem to be enzymic, since it occurs with great rapidity. There is no other evidence of the presence of an enzyme in the liver of the rat which can accomplish this reduction, as shown by the slowness of sulphate-formation from cystine. Therefore the evidence seems to indicate that cystine itself may be the immediate substrate in the oxidation to cysteic acid. Cysteinesulphinic acid is oxidized by the same enzyme, or by a second enzyme present in the same preparation. It may therefore be a stage in the oxidation of cysteine to cysteic acid. An attempt will be made to settle some of these problems with more highly purified enzyme preparations.

SUMMARY

Four enzymes have been extracted from liver tissue of the albino rat.

1. Cytochrome oxidase, which oxidizes cysteine quantitatively to cystine. It has no effect on methionine or cysteinesulphinic acid.

2. An enzyme which oxidizes the sulphur of cysteine to inorganic sulphate. An active crude liver brei has been obtained but attempts at further purification proved unsuccessful. It is possible that this enzyme carries the oxidation only to the sulphinic acid stage. Disulphides cannot act as substrates.

3. An enzyme which oxidizes the sulphur of cysteinesulphinic acid to inorganic sulphate.

4. An enzyme which oxidizes cysteine to cysteic acid (according to iodimetric titration). This oxidation probably represents the first step in the production of taurine. Cysteinesulphinic acid is possibly an intermediate.

The author is indebted to Anna Katherine Stimson for help in the preparation of the charts and of some of the material.

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CXCII. METABOLISM OF SULPHUR

IX. THE NON-PRODUCTION OF GLYCOGEN FROM CYSTINE, CYSTEINE AND METHIONINE AND THEIR PARTIALLY OXIDIZED DERIVATIVES¹

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THE recent work of Butts *et al.* [1938], showing that the feeding of cystine to normal fasting rats does not lead to glycogen deposition in the liver, has made it desirable that the fate of methionine and cysteine in this respect be investigated also. A separate study of these amino-acids is especially important since there is evidence that methionine and cysteine do not always follow the same metabolic pathway as cystine. In this investigation possible glycogen production from some of the partially oxidized derivatives of cystine and methionine has been included, since it has been shown that their fate in metabolism also differs from that of the parent amino-acids in that their administration leads to inorganic sulphate excretion to a less degree than does consumption of the disulphide or thiol amino-acids.

In general, the technique employed by Butts *et al.* was followed. The rats were starved for 36 hr., during which time they had free access to filter paper. The solutions were then administered in four equal portions at hourly intervals. A small catheter attached to a syringe was used as a stomach tube. One hour after the fourth feeding the rat was killed by a blow on the head, the liver was removed immediately, dropped into previously weighed alkali and the tubes re-weighed. Liver glycogen was determined by the method of Good *et al.* [1933].

The following is a list of the compounds employed, together with their modes of preparation:

Cystine was administered in gum tragacanth as described by Butts *et al.*

Sodium cystinate and *methionine sulphoxide* were administered in aqueous solution.

Methionine was dissolved in 10–12 drops of conc. HCl and the solution neutralized to Congo red with NaOH.

Cysteinesulphinic acid and *cystic acid* were dissolved in water and neutralized to Congo red.

The amount fed, following the procedure of Butts *et al.*, was 0.0178 m.-equiv. of S per cm.² body surface. The surface area was estimated from the formula $S = kW^{2/3}$, where $k = 9.1$ [Wilson & Lewis, 1929]. The total volume in each case was about 15 ml. The results are expressed in g. glycogen per 100 g. liver and are given in the last column of Table I. Each glycogen figure is the average of two or usually three duplicate samples.

The results with cystine agree with those of Butts *et al.* in that the percentages of glycogen in the livers do not differ significantly from those of the controls which were starved without subsequent feeding of amino-acid, and the figures for alanine are of about the same order as those obtained by them. Another rat

¹ Aided by a grant from the Blanche and Frank Wolf Foundation, Inc.

Table I. *Glycogen in the liver of albino rats following feeding of sulphur-containing amino-acids and their partially oxidized derivatives*

Exp. no.	Wt. of rat g.	Period starved hr.	No. of doses	Interval before killing hr.	Substance fed	Amount fed g.	Glycogen in liver %
1	—	—	—	—	—	—	7.278
2	—	24	—	—	—	—	0.114
3	455	36	4	1	Glucose	5.0	3.953
4	399	36	4	1	Alanine	0.781	1.908
5	357	36	4	1	Alanine	0.726	2.436
6	335	36	4	1	<i>l</i> -Cystine	0.945	0.065
7	332	36	4	1	<i>l</i> -Cystine	0.940	0.072
8	353	36	4	1	<i>Na-l</i> -cystinate	1.164	0.086
9	347	36	4	1	<i>dl</i> -Methionine	1.192	0.070
10	336	36	4	1	<i>dl</i> -Methionine	1.166	0.099
11	248	36	4	1	<i>dl</i> -Methionine	0.953	0.079
12	372	36	4	1	<i>l</i> -Cysteine	1.014	0.077
13	287	36	4	1	<i>l</i> -Methionine sulfoxide	1.163	0.068
14	283	36	4	1	<i>l</i> -Methionine sulfoxide	1.152	0.069
15	368	36	4	1	<i>l</i> -Cystenesulphinic acid	1.273	0.070
16	360	36	4	1	<i>l</i> -Cysteic acid	1.385	0.053
17	305	36	4	1	<i>l</i> -Cysteic acid	1.240	0.078
18	332	24	1	6	<i>dl</i> -Methionine	1.157	0.115
19	242	24	1	6	<i>dl</i> -Methionine	0.937	0.105
20	335	36	1	6	<i>l</i> -Cysteine	0.945	0.114

(animal 3) was employed, feeding glucose as a further test of the method. The results show that none of the sulphur-containing amino-acids fed under these conditions gave rise to glycogen in the liver. In this respect, then, cystine, methionine and cysteine agree metabolically.

Although cystenesulphinic acid and cysteic acid give rise to taurine in the bile [Virtue & Doster-Virtue, 1939] and produce less inorganic sulphate in the urine than the corresponding naturally occurring amino-acids, nevertheless there is a slight rise of inorganic sulphate in the urine following their ingestion, and it was thought that at least these molecules might contribute to glycogen formation. But the results indicate otherwise.

Stöhr [1938] recently published a table in which the results of feeding cystine and cysteine differed radically from these. His method also differed somewhat in that the substance to be fed was given at one dose, followed by an interval of 6 hr. before the rat was killed. He used this procedure since he had evidence that repeated feedings accelerated the resorption of glycogen from the liver, so that lower results were obtained. In the case of cysteine he obtained a liver glycogen of 0.584 ± 0.111 %, as contrasted with 0.071 ± 0.009 % in the controls. When he used *d*-alanine, 0.524 ± 0.075 % was found. We therefore repeated three experiments using his procedure, one with cysteine and two with methionine. They are recorded in Table I as exps. 18 and 19 (methionine) and 20 (cysteine). As may be seen, while the glycogen percentages in the liver obtained by his procedure are slightly higher than those given by the method used above, they are not more elevated than our controls and are considerably lower than the corresponding figures given by him.

SUMMARY

Cystine, methionine and cysteine, fed to rats starved for 24 or 36 hr. before receiving 0.0178 m.-equiv. of the compound per cm.² body surface, fed in four hourly portions, do not give rise to glycogen in the liver, as shown by analysis

1 hr. after the final feeding. Methionine sulphoxide, cysteinesulphinic acid and cysteic acid also do not lead to glycogen deposition in the liver under these conditions.

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CXCIII. CYTOCHROME *c* AS A CARRIER WITH THE GLUCOSE DEHYDROGENASE SYSTEM

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A FEW dehydrogenases of the aerobic oxidase type can apparently catalyse the direct oxidation of their substrates by molecular oxygen, but in the majority of cases the aerobic oxidation of metabolites by dehydrogenase systems occurs only in presence of some suitable carrier system to act as an intermediary between oxygen and the hydrogen of the substrate undergoing oxidation.

The first experiments with cytochrome *c* as carrier with a dehydrogenase system *in vitro* were carried out by Harrison [1931], who showed that addition of cytochrome *c* and cytochrome oxidase to the glucose dehydrogenase preparation obtained by him from liver resulted in a system capable of bringing about the aerobic oxidation of glucose. Ogston & Green [1935], however, were unable to confirm Harrison's results with glucose dehydrogenase and concluded from their experiments that of a number of dehydrogenase systems tested only the succinic dehydrogenase and the yeast lactic dehydrogenase reacted with the cytochrome *c*-cytochrome oxidase system. Green [1936] later showed that the α -glycerophosphoric dehydrogenase is similar in this respect to these two dehydrogenases.

As a result of Ogston & Green's work, Green & Brosteaux [1936] suggested that the group of coenzyme dehydrogenases (which includes glucose dehydrogenase) was unable to utilize the cytochromes as carriers. Recently, however, Dewan & Green [1938] have demonstrated that the aerobic oxidation of substrates by several coenzyme dehydrogenases can occur with cytochromes *a* and *b* as carriers, provided that in addition to the cytochromes and cytochrome oxidase another factor (diaphorase) is present to act as intermediary between the coenzyme and the cytochromes. However, in these experiments also, addition of cytochrome *c* to the combined enzyme systems had no effect on the rate of oxidation of the substrate, and Dewan & Green therefore concluded that the aerobic oxidation of substrates by coenzyme dehydrogenases is normally brought about with the diaphorase-cytochrome *a* and *b*-cytochrome oxidase system as carrier mechanism, and that cytochrome *c* is unable to act as intermediate carrier with such dehydrogenase systems.

In the course of an investigation of the oxidation of glucose by enzyme systems present in animal tissues we have found, as might be expected from Dewan & Green's work, that the aerobic oxidation of glucose is brought about by the glucose dehydrogenase system *plus* the cytochrome *a* and *b* system. In carrying out this work we had an opportunity to reinvestigate the carrier action of cytochrome *c* with the glucose dehydrogenase and have found that under suitable conditions addition of cytochrome *c* to the combined dehydrogenase and cytochrome oxidase systems does increase the O_2 uptake due to glucose oxidation. Further, we have been able to show spectroscopically that the glucose dehydrogenase system can reduce cytochrome *c*. Harrison's claim that cytochrome *c*

acts as carrier with this dehydrogenase system is therefore substantiated. In the course of these experiments evidence has been obtained that some factor, which may be the coenzyme factor diaphorase, is necessary for the reduction of cytochrome *c*, and it seems possible that a deficiency of this factor in the oxidase preparations of Ogston & Green may explain the inability of these workers to confirm Harrison's results.

EXPERIMENTAL

(1) *Preparation of material*

Glucose dehydrogenase was prepared from ox liver by the second method of Harrison [1933], the precipitation by ammonium sulphate being repeated a number of times until a preparation sufficiently free from residual substrates was obtained. The enzyme solutions were prepared by dissolving a weighed amount of the dry preparation as completely as possible in phosphate buffer and centrifuging off inactive material. The activity of the solutions was always tested by the Thunberg methylene blue technique before use in the aerobic experiments.

Coenzyme I (cozymase) prepared from baker's yeast by the method of Green *et al.* [1937] was used instead of the coenzyme preparation from liver described by Harrison.

Cytochrome *c* was extracted from ox heart by the method of Keilin & Hartree [1937]; the precipitate obtained with 20 % trichloroacetic acid, after washing with saturated ammonium sulphate solution, was dissolved in ammonia (2 ml. of concentrated ammonia solution diluted to 400 ml.) and dialysed in parchment sacs in the ice-chest for 2 days against frequent changes of dilute ammonia. After removal of the ammonia *in vacuo* the residue was dissolved in water and diluted to a strength of 3×10^{-4} *M*. A number of different preparations were used in this work and fresh solutions were made up at frequent intervals.

In most cases cytochrome oxidase prepared from pig heart by the method of Keilin & Hartree [1938] was used. The preparations were purified by repeating three times the precipitation from phosphate by addition of acetate buffer. The preparation probably contained little if any cytochrome *c* since neither the final supernatant fluid, the preparation itself, nor a trichloroacetic acid extract of the preparation made by the Keilin & Hartree method for preparing cytochrome *c* showed any absorption bands of reduced cytochrome *c* on addition of sodium hydrosulphite. Stock solutions of the oxidase were made by suspending 5.6 g. of the preparation in *M*/10 phosphate buffer pH 7.4 and diluting to 10 ml. These solutions were stored in the ice chest and were diluted with phosphate buffer to the required strength before use. Fresh preparations of oxidase were made up every few days.

The aerobic experiments were carried out in Barcroft differential manometers. The various solutions, diluted to a total volume of 3 ml., were placed in the right-hand flasks, while the left-hand flasks received an equal volume of distilled water. In the centre compartment of each flask was placed 0.2 ml. of 5 % NaOH soaked on a roll of filter paper to absorb any CO₂, except in the experiments on cyanide inhibition, when, in order to avoid errors due to absorption of HCN by the NaOH, the solutions recommended by Krebs [1935] were used instead. The manometers were shaken at 100 oscillations per min. at a constant temperature of 37°.

Anaerobic experiments were carried out in Thunberg tubes which were evacuated and filled with N₂ twice, then re-evacuated and incubated at 37°.

(2) *Aerobic oxidation of glucose with the cytochromes as carriers*

The oxidation of glucose by the glucose dehydrogenase system *plus* the diaphorase-cytochrome *a* and *b*-cytochrome oxidase system, and the effect of addition of cytochrome *c* are shown in Table I.

Cytochrome oxidase preparations made by the Keilin & Hartree method, although probably free from cytochrome *c* (as indicated by the failure to detect the absorption bands of reduced cytochrome *c* on treatment with hydrosulphite) contain cytochromes *a* and *b* and diaphorase in addition to cytochrome oxidase.

Table I. *Glucose oxidation in presence of the cytochromes*

No. of manometer	1	2	3	4	5	6	7	8
Dehydrogenase (ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glucose (ml.)	—	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cytochrome <i>c</i> (ml.)	1.0	1.0	—	1.0	—	1.0	—	1.0
Oxidase (ml.)	0.2	—	0.2	0.2	0.2*	0.2*	0.2†	0.2†
Distilled water (ml.)	0.3	0.2	1.0	—	1.0	—	1.0	—
O ₂ absorbed (μl) after 90 min.	19	14	751	750	297	485	188	274

The dehydrogenase solution contained 55 mg. of the dry preparation (equivalent to 5 g. acetone liver) in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 4 mg. per ml.; glucose, 2*M*; cytochrome *c*, 3×10^{-4} *M*; oxidase, Keilin & Hartree's preparation, stock solution; * diluted 1:20 with *M*/10 phosphate buffer pH 7.4; † diluted 1:40.

The activity of the dehydrogenase was such that 0.2 ml. of the enzyme solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (in a total volume of 1.65 ml.) in 5 min., while without coenzyme or without glucose the times of reduction were 30 min. and 35 min. respectively.

As was expected from Dewan & Green's work, addition of such an oxidase preparation to a mixture of glucose dehydrogenase, coenzyme I and glucose forms a complete enzyme system capable of bringing about the aerobic oxidation of the glucose. This is shown by comparing manometer 3 (or manometers 5 or 7) with the controls, manometers 1 and 2. Comparing the O₂ uptakes in the pairs of manometers 3 and 4 in which a high concentration of the oxidase-cytochrome *a* and *b* preparation was used, it can be seen that addition of cytochrome *c* produced no increase in O₂ uptake. On the other hand, comparing manometers 5 and 6 where a much more dilute oxidase preparation was added, it is evident that in this case addition of cytochrome *c* resulted in a large increase in the amount of O₂ absorbed as compared with the amount absorbed by the combined dehydrogenase-cytochrome *a* and *b* system without cytochrome *c*. A similar result was obtained on further decreasing the amount of oxidase-cytochrome *a* and *b* preparation used as can be seen by comparing manometers 7 and 8. Apparently, therefore, in presence of relatively large amounts of the oxidase-cytochrome *a* and *b* preparation, the total hydrogen transport proceeds via cytochromes *a* and *b*, since under these conditions addition of cytochrome *c* has no effect on the rate of oxidation of glucose. If, however, the amount of cytochrome oxidase preparation added is decreased so that the concentration of the cytochrome carrier becomes the limiting factor in the reaction then addition of cytochrome *c* leads to an increased O₂ uptake. Under these conditions, therefore, cytochrome *c* can act as carrier with the glucose dehydrogenase system.

The effect of cytochrome *c* in increasing the rate of oxidation of glucose by the dehydrogenase system *plus* the cytochrome *a* and *b* and oxidase system has

been confirmed in a large number of experiments, the results of some of which are summarized in Table II. The final amounts of O_2 absorbed by the following four systems are shown:

A	B	C	D
Dehydrogenase	Dehydrogenase	Dehydrogenase	Dehydrogenase
Coenzyme	Coenzyme	Coenzyme	Coenzyme
—	Glucose	Glucose	Glucose
Cytochrome <i>c</i>	Cytochrome <i>c</i>	—	Cytochrome <i>c</i>
Oxidase	—	Oxidase	Oxidase

Table II. *Glucose oxidation in presence of cytochrome c*

Time of experiment min.	Dilution of stock oxidase	Oxygen uptakes (μ l.)			
		A	B	C	D
120	1:20	33	36	266	388
90	1:10	33	26	417	543
135	1:15	49	68	379	588
180	1:15	79	79	279	564
180	1:20	25	24	382	551
75	1:15	37	49	371	520
120	1:60	21	38	190	344
150	1:30	25	24	361	499

The conditions varied slightly in different experiments but as a general rule the following quantities were used: dehydrogenase (55–90 mg.) equivalent to 5 g. acetone liver dissolved in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 2 mg.; glucose, final concentration 0.2 *M*; cytochrome *c*, final concentration 10^{-4} *M*; oxidase, 0.2 ml. of a solution prepared by diluting the stock solution with *M*/10 phosphate buffer pH 7.4 as shown in the second column. The activities of the dehydrogenase solutions used were such that 0.2 ml. dehydrogenase + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (in a total volume of 1.65 ml.) in from 5 to 7 min., while without glucose or without coenzyme the time required for the reduction of the methylene blue was at least five times as great.

In many cases controls without dehydrogenase or without coenzyme were set up in addition to the above. Actually the O_2 uptake in the systems either without glucose or without oxidase (A and B respectively) was found to be due almost entirely to the dehydrogenase solution alone, and this figure is therefore common to all four systems. It can be seen from Table II that while addition of the cytochrome *a* and *b*-cytochrome oxidase preparation alone to the dehydrogenase system resulted in an aerobic oxidation of glucose (column C), addition of cytochrome *c* led to a further increase in the amount of O_2 absorbed (column D). Exactly similar results have been obtained using cytochrome oxidase preparations made from sheep heart by a slight modification of the older method of Keilin [1930], or preparations from pig heart made by the method used by Dewan & Green [1938] for preparing "coenzyme factor".

The increase in O_2 uptake on addition of cytochrome *c*, like the O_2 uptake which results on adding the cytochromes *a* and *b*-cytochrome oxidase preparation alone to the dehydrogenase system is completely inhibited by *M*/1000 cyanide as shown in Table III.

Comparing manometers 5 and 6 with manometers 3 and 4 it is clear that cyanide completely inhibits the oxidation of glucose, the O_2 uptakes with cyanide being reduced to those of the controls without glucose or without oxidase (manometers 1 and 2). As is well known the activity of the cytochrome oxidase system is inhibited by dilute cyanide. This experiment affords evidence, therefore, that the oxidation of glucose is brought about by the cytochromes and not by other carriers present in the enzyme preparations.

Table III. *Inhibition by cyanide of glucose oxidation*

No. of manometer	1	2	3	4	5	6	7
Dehydrogenase (ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
2 <i>M</i> glucose (ml.)	—	0.3	0.3	0.3	0.3	0.3	0.3
Cytochrome <i>c</i> (ml.)	1.0	1.0	—	1.0	—	1.0	—
Oxidase (ml.)	0.2	—	0.2	0.2	0.2	0.2	—
<i>M</i> /100 KCN (ml.)	—	—	—	—	0.3	0.3	1.0
1/500 methylene blue (ml.)	—	—	—	—	—	—	0.5
Distilled water (ml.)	0.6	0.5	1.3	0.3	1.0	—	—
Oxygen absorbed (μ l.) after 180 min.	48	43	312	418	39	49	429

The dehydrogenase solution contained 50 mg. (equivalent to 5 g. acetone liver) in 1 ml. *M*/10 phosphate buffer pH 7.4; coenzyme I, 10 mg. per ml.; cytochrome *c*, 3×10^{-4} *M*; oxidase prepared by the Keilin & Hartree method, diluted 1:20. The cyanide was brought to pH 7.4 with HCl and made up to *M*/100.

The activity of the dehydrogenase was such that 0.2 ml. of the solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue (total volume 1.65 ml.) in 8 min., while without coenzyme or without glucose more than an hour was required.

This result is not due to inhibition of the dehydrogenase itself by cyanide, for cyanide in final concentration of even *M*/300 does not inhibit the activity of the dehydrogenase as is shown by adding methylene blue as carrier. It can be seen from the last column of Table III that with methylene blue added the full O_2 uptake is obtained even in presence of cyanide.

In his original experiments, on the aerobic oxidation of glucose by glucose dehydrogenase *plus* cytochrome *c* and cytochrome oxidase, Harrison noted the O_2 uptake caused on addition of cytochrome oxidase alone to the dehydrogenase system. This he attributed to small amounts of cytochrome *c* in the oxidase preparations, whereas it now seems clear from the above experiments, taken in conjunction with the work of Green and his co-workers, that it is due to the cytochromes *a* and *b* present in the oxidase preparation. The claim by Harrison, however, that cytochrome *c* can act as carrier with the glucose dehydrogenase system is completely confirmed by this investigation.

It is surprising that Ogston & Green were not only unable to repeat Harrison's results with cytochrome *c* but observed no O_2 uptake on addition of cytochrome oxidase preparations alone to any of the coenzyme I-dehydrogenase systems tested. Since these authors themselves pointed out that the cytochrome oxidase preparations used by them contained also cytochromes *a* and *b*, the only explanation of their results appears to be that the preparations were deficient in diaphorase, the factor which is required as intermediate carrier between the coenzyme and the cytochromes. We carried out some experiments similar to those described above, using the indophenol (cytochrome) oxidase preparations described in Ogston & Green's paper, but the results were unsatisfactory: in some cases an O_2 uptake on addition of the cytochrome oxidase to the glucose dehydrogenase system was observed, but it was always considerably smaller than that obtained with an oxidase preparation made from the same heart by Keilin's method.

(3) *Anaerobic reduction of cytochrome c by the glucose dehydrogenase system*

The ability of cytochrome *c* to act as carrier with the glucose dehydrogenase system was confirmed by studying the reduction of cytochrome *c* by the system under anaerobic conditions.

A mixture of glucose dehydrogenase, coenzyme I and glucose rapidly reduces methylene blue under anaerobic conditions, but although glucose dehydrogenase

has a very low (negative) reduction potential and therefore from the thermodynamic point of view would be expected to be capable of reducing the much more positive cytochrome *c*, yet if the methylene blue in the above system is replaced by cytochrome *c*, only very slight reduction of the cytochrome actually occurs. This reduction is independent of both glucose and coenzyme I and is probably due to small amounts of other enzyme systems in the dehydrogenase preparation. It was found, however, that in presence of an additional factor contained in cytochrome oxidase preparations made by either the Keilin or the Keilin & Hartree method, the cytochrome *c* is rapidly and fully reduced by the glucose dehydrogenase system. This is shown in Table IV in which is given the time required for complete reduction of the cytochrome *c*.

Table IV. *Anaerobic reduction of cytochrome c*

No. of Thunberg tube	1	2	3	4	5
Dehydrogenase (ml.)	—	1.0	1.0	1.0	1.0
Coenzyme I (ml.)	0.2	—	0.2	0.2	0.2
Glucose (ml.)	0.2	0.2	—	0.2	0.2
Oxidase (factor) (ml.)	0.2	0.2	0.2	—	0.2
Cytochrome <i>c</i> (ml.)	0.5	0.5	0.5	0.5	0.5
Time (min.)	>30	>30	>30	>30	5

The dehydrogenase solution contained 9 mg. of the dry preparation (equivalent to 1 g. acetone liver) in 1 ml. *M*/20 phosphate buffer pH 7.4; coenzyme I, 4 mg. per ml.; glucose, 2*M*; cytochrome *c*, 3×10^{-4} *M*; oxidase, stock solution prepared by Keilin's method. The total volume in each tube was made up to 2.1 ml. by addition of distilled water where required.

In this experiment, in order to decrease the residual reducing power of the dehydrogenase solution as far as possible, a mixture of 10 ml. of the dehydrogenase solution, 1 ml. cytochrome *c* and 0.5 ml. of cytochrome oxidase was placed in a water bath at 37° and aerated for 20 min. to allow residual substrates to become oxidized. The cytochrome oxidase was then removed by centrifuging for 10 min. The activity of the dehydrogenase was then tested with methylene blue before use in the experiments on the reduction of cytochrome. 1 ml. of the dehydrogenase solution + 0.2 ml. coenzyme I (4 mg. per ml.) + 0.2 ml. 2*M* glucose reduced 0.25 ml. 1/5000 methylene blue in 8 min., while without glucose or without coenzyme more than 30 min. were required.

The reduction of the cytochrome *c* was carried out in Thunberg tubes which were evacuated and filled with nitrogen twice, re-evacuated and incubated at 37°. Reduction of the cytochrome was easily followed by the change in colour. For comparative purposes two standard tubes were used, the first containing a sample of cytochrome *c* reduced by hydrosulphite to which were added the other components as in tube 5, while the second contained a system identical with that in tube 5 but incubated under aerobic conditions so that the cytochrome was maintained in the oxidized form by the cytochrome oxidase. In addition, the solutions were examined spectroscopically before opening the tubes. After 5 min. incubation the solution in tube 5 showed strong bands of reduced cytochrome *c*, while in the other tubes the α -band of reduced cytochrome *c* could only just be detected after 30 min. incubation. This experiment has been repeated a number of times with similar results.

It is clear, therefore, that cytochrome *c* is not directly reduced by the dehydrogenase system but that the reaction is catalysed by a factor contained in heart muscle and found in cytochrome oxidase preparations made from it. (In these experiments the cytochrome oxidase as such plays no part, of course, in the reaction since this is carried out under anaerobic conditions. The oxidase preparation is added solely as source of the factor.)

In view of the well-known effect of yeast flavoprotein in catalysing the reduction by coenzyme dehydrogenase systems of carriers such as methylene blue it seemed possible that the factor required for the reduction of cytochrome *c* might be identical with this Warburg-Christian flavoprotein. We found, however, that while flavoprotein prepared from yeast showed some catalytic action, it was very much less active than the cytochrome *c* factor in the oxidase preparations. Since these experiments were completed, diaphorase, the factor required for the reduction of cytochromes *a* and *b* by dehydrogenase systems, has been identified with a flavoprotein isolated by Straub [1939, 1, 2] from heart muscle [Straub *et al.* 1939; Corran *et al.* 1939] and it is interesting that Corran *et al.* have found that yeast flavoprotein has a small, though relatively negligible, effect in the cytochrome *a* and *b* system.

It seems possible that the factor required for the reduction of cytochrome *c* by the glucose dehydrogenase is identical with diaphorase. On the other hand, Hopkins *et al.* [1939] and Dixon & Zervas [1939] have demonstrated that the reduction of cytochrome *c* by the succinic dehydrogenase and yeast lactic dehydrogenase occurs only in presence of a factor which is probably not diaphorase, and Potter & Lockhart [1939] have recently suggested in the case of the succinic enzyme that the factor may be cytochrome *b*. Until more evidence is available, it appears undesirable, therefore, to speculate further on the nature of the factor required with the glucose dehydrogenase system.

SUMMARY

Cytochrome *c* in presence of cytochrome oxidase preparations can act as a carrier and bring about the aerobic oxidation of glucose by the glucose dehydrogenase system. The cytochrome oxidase preparations can, by themselves, induce some oxidation of glucose in presence of the dehydrogenase system. This is apparently due to the other carriers, cytochromes *a* and *b*, present in the oxidase preparations.

In acting as a carrier in this oxidation cytochrome *c* is not directly reduced by the dehydrogenase-coenzyme system but requires for its reduction an additional factor which is present in heart muscle and in the cytochrome oxidase preparations made from heart muscle.

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CXCIV. THE HYDROLYSIS OF GLUCOSAMINIDES BY AN ENZYME IN *HELIX POMATIA*

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It has long been known that the polysaccharide chitin when completely hydrolysed yields glucosamine and acetic acid; in recent years it has indeed been shown conclusively that the basic unit of the polysaccharide is *N*-acetylglucosamine. The evidence for the latter statement rests, firstly, on the discovery by Karrer & Hofmann [1929] of an enzyme in *Helix pomatia* which is able to hydrolyse chitin to *N*-acetylglucosamine and, secondly, on the isolation of chitobiose octaacetate by Bergmann *et al.* [1931] and by Zechmeister & Toth [1931] as a product of acetolysis of chitin.

In view of the conclusions which have been reached concerning the structure of polysaccharides from experiments on their hydrolysis by enzymes possessing α - or β -specificity, it was thought that an enzyme such as that discovered by Karrer might, if it could be characterized, be used to investigate the stereochemical structure not only of chitin itself but of other glucosamine-containing polysaccharides such as that of egg albumin [Neuberger, 1938]. For this purpose it was clearly necessary to determine the α - or β -specificity of the enzyme before any conclusions could be reached as to the configuration of the natural linkages in such polysaccharides, and it was also thought desirable to investigate other factors which might influence enzyme action, such as variations in the *N*-acyl group and substitution on carbon atoms other than C₁.

Up to the present, little has been known about the specificity of chitin-splitting enzymes. Zechmeister and his co-workers [1932] found that emulsin contains an enzyme capable of hydrolysing chitodextrin, a product obtained by partial acid hydrolysis of chitin. It was first presumed by these authors that the enzyme responsible for hydrolysis was the glucosidase in emulsin which is known to possess β -specificity, and they therefore concluded that chitodextrin and chitin have a β -structure. The assumption they made was shown to be incorrect by Helferich & Iloff [1933], who found that β -glucosidase and chitinase were different enzymes. Later, Zechmeister and his co-workers [1934] confirmed Helferich's observation, by demonstrating that a highly purified sample of β -glucosidase is inactive towards chitin.

With regard to the *N*-acyl specificity of the enzyme, some preliminary experiments were done by Karrer & White [1930]; these authors treated chitin with strong alkali and obtained a poorly characterized but presumably partly deacetylated product, chitosan, which, on enzymic hydrolysis, yielded a mixture. If, however, the chitosan were reacetylated, the product of enzymic hydrolysis was pure *N*-acetylglucosamine. Products purporting to be *N*-formyl, *N*-propionyl, *N*-butyryl and *N*-benzoyl derivatives of chitosan were also prepared, of which the only one to be attacked by the enzyme was the *N*-benzoyl derivative, and that to only a small extent. These experiments suggest that the enzyme will

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not attack linkages between free glucosamine residues and is indeed specific towards *N*-acetyl compounds, but the matter cannot be regarded as settled; no analytical evidence bearing on the composition of the reacylated derivatives was presented and in so far at least as the formyl derivative is concerned, Karrer's conditions of formylation were not such as, in our experience, would lead to any acylation.

Further work on the *N*-acetyl specificity of this type of enzyme was done by Helferich *et al.* [1934], who examined the action of emulsin on phenyl glucosaminides. They found that emulsin contains an enzyme which hydrolyses *N*-acetyl-phenylglucosaminide, although it will not hydrolyse the corresponding unacylated compound; this latter observation is in agreement with that of Irvine & Hynd [1913] who found that emulsin cannot hydrolyse methylglucosaminide hydrochloride. This experiment provides further evidence that the enzyme will only hydrolyse acylated glucosamine derivatives. As regards the configuration of Helferich's phenylglucosaminide, the method of preparation and the rotation indicate that it has a β -configuration, but since no corresponding α -compound was available for purposes of comparison, this matter cannot be decided with any certainty.

Since a pair of *N*-acetyl methylglucosaminides were prepared by the authors [1939] it became possible to discover whether one of the enzymes capable of hydrolysing glucosaminides showed either α - or β -specificity, and the *Helix pomatia* enzyme was chosen for this purpose. In order to investigate the *N*-acetyl specificity of the enzyme, various *N*-acylated glucosaminides were prepared, and the effect of the enzyme on these, as well as on *O*-substituted glucosaminides already described in the literature, was examined.

EXPERIMENTAL

(1) Preparation of compounds

N-*p*-Toluenesulphonyl tetra-acetylglucosamine. Tetra-acetylglucosamine was prepared according to Bergmann & Zervas [1931] from 3.83 g. of tetra-acetylglucosamine hydrochloride, and dissolved in 20 ml. dry pyridine. *p*-Toluenesulphonyl chloride (1.9 g.), recrystallized from ligroin, was dissolved in pyridine and the two solutions were mixed, left at room temperature for 2½ hr. and heated for 30 min. on a boiling water bath; the solution was then poured into iced water and extracted with chloroform. The chloroform solution was washed with *N* HCl and water, dried over sodium sulphate and evaporated to dryness under diminished pressure. The substance crystallized from 30% methyl alcohol in needles. Yield 56%. M.P. 128–129°. $[\alpha]_D - 3$ in chloroform. (Found: N, 2.8%; $C_{21}H_{27}O_{11}NS$ requires N, 2.8%.)

1: *Bromo-N-p-toluenesulphonyl triacetylglucosamine*. *N-p*-Toluenesulphonyl tetra-acetylglucosamine (1 g.) was dissolved in glacial acetic acid (10 ml.) saturated at 0° with HBr. The solution was cooled to 0° and HBr was passed in for 45 min. It was then left for 2 hr. at room temperature, poured on to crushed ice and extracted with chloroform. The chloroform solution was washed with ice-cold bicarbonate solution till neutral to litmus, and once with brine, and dried over sodium sulphate. The chloroform was removed under diminished pressure and finally *in vacuo*. Crude yield 83%. The compound crystallized from glacial acetic acid and dry ether in needles. M.P. 148°. $[\alpha]_D + 63.5$ (in chloroform). (Found: C, 43.74; H, 4.83; Br, 15.2%. $C_{18}H_{24}O_9NSBr$ requires C, 43.70; H, 4.60; Br, 15.33%.)

N-p-Toluenesulphonyl triacetylphenylglucosaminide. Sodium (0.77 g.) was dissolved in dry phenol (18.2 g.) and 1:bromo-*N-p*-toluenesulphonyl triacetylglucosamine (1.86 g.) was added. The solution was heated on a water bath with exclusion of moisture for 1 hr. with continuous shaking, and poured into cold water. The oil presently solidified; it was washed several times with cold water, and filtered. The compound crystallized from absolute alcohol in needles. Yield 1.5 g. M.P. 200–201°. $[\alpha]_D - 52.8^\circ$ in pyridine. (Found: N, 2.7%; $C_{25}H_{29}O_{10}NS$ requires N, 2.6%.)

N-p-Toluenesulphonyl phenylglucosaminide. *N-p*-Toluenesulphonyl triacetyl- β -phenylglucosaminide (0.535 g.; 1 millimol.) was dissolved in 10 ml. absolute methyl alcohol, 0.5 millimol. barium methoxide was added and the mixture was left at room temperature for 2 hr. The solution was exactly neutralized with 5*N* H_2SO_4 and filtered. The barium sulphate was extracted twice with absolute alcohol and the combined solutions were evaporated to a low volume and cooled. Yield 91%. The compound crystallized from absolute alcohol in needles. M.P. 213–214°. $[\alpha]_D - 83^\circ$ in pyridine. (Found: C, 55.57; H, 5.705; N, 3.43%. $C_{19}H_{27}O_7NS$ requires C, 55.61; H, 5.854; N, 3.4%.)

It was desired to obtain an *N*-formylglucosaminide, and attempts were made to prepare both β -methyl and β -phenyl triacetyl *N*-formylglucosaminides by heating the corresponding free bases with formic acid distilled under diminished pressure from anhydrous copper sulphate. No formyl compound was obtained. It was found, however, that if β -methyl triacetylglucosaminide formate is heated to 155° for 3 hr. the pure formyl compound is obtained in very good yield.

N-Carbobenzyloxy triacetyl- β -methylglucosaminide [Neuberger & Pitt Rivers, 1939]. *N*-Carbobenzyloxy- β -methylglucosaminide (1 g.) was dissolved in dry pyridine (10 ml.) and dry acetic anhydride (8 ml.) was added. The mixture was left for 14 hr. at 2°, poured on to crushed ice, filtered and washed with ice-cold water. The substance crystallized from 30% methyl alcohol in large needles. Yield 1.2 g. (87%). M.P. 147–149°. $[\alpha]_D + 15^\circ$ in chloroform. (Found: N, 3.09%. $C_{21}H_{28}ON$ requires N, 3.08%.)

Triacetyl- β -methylglucosaminide formate. *N*-Carbobenzyloxy triacetyl- β -methylglucosaminide (0.5 g.) was dissolved in 15 ml. dry formic acid and catalytically reduced in the presence of Pd black. The formic acid was removed under diminished pressure, chloroform was added and the compound isolated by precipitation with ether. Yield 0.3 g. (75%). M.P. 120°. 18.2 mg. of this compound required 2.5 ml. *N*/50 NaOH using bromothymol blue as indicator (calculated, 2.49 ml.).

N-Formyl triacetyl- β -methylglucosaminide. Triacetyl- β -methylglucosaminide formate (0.5 g.) was heated to 155° in boiling anisole for 3 hr. The anisole was removed under diminished pressure at 60°. Yield 0.25 g. M.P. 165°. (Found: N, 4.12%. $C_{14}H_{21}O_9N$ requires N, 4.03%.)

N-Formyl- β -methylglucosaminide. *N*-Formyltriacetyl- β -methylglucosaminide (0.25 g.) was dissolved in 0.2 ml. water and treated with 3 equiv. of aqueous baryta. The solution was left at room temperature for 2 hr., exactly neutralized with H_2SO_4 and centrifuged. The water was removed under diminished pressure at 35°. Alcohol was added several times and evaporated off. The substance crystallized from absolute alcohol and ether in needles. Yield 0.149 g. (93%). M.P. 204–205°. $[\alpha]_D - 47.2^\circ$ in water. (Found: C, 43.17; H, 7.58; N, 6.2%. $C_8H_{15}O_6N$ requires C, 43.43; H, 6.8; N, 6.33%.)

It will be observed that the analysis of this compound is unsatisfactory in respect of the hydrogen; a similarly high figure for the hydrogen was obtained by an independent analyst. In spite of this, however, we feel no doubt concerning

the identity of the substance in view of the following experiments: (a) hydrolysis of a small amount with 5*N* HCl for 20 hr. gave an almost theoretical yield of glucosamine hydrochloride having $[\alpha]_D + 73^\circ$ (equilibrium value in water) and *N* 6.3%; (b) analysis by the method of Pregl & Soltys [1929] gave volatile acid amounting to 13.09% calculated as formic acid (theory 13.1%); moreover the distillate after neutralization and concentration reduced ammoniacal silver nitrate on warming.

N-Propionyl-triacetyl- β -phenylglucosaminide. Triacetyl- β -phenylglucosaminide hydrochloride (0.417 g.) was suspended in chloroform (20 ml.); the suspension was cooled in ice and triethylamine (0.32 ml.) was added. Propionyl chloride (0.12 ml.) was added and the solution left at room temperature for 1 hr. Chloroform (20 ml.) was added and the chloroform solution was washed successively with *N* HCl, bicarbonate solution and water, and dried over sodium sulphate. The chloroform was removed under diminished pressure. Yield 78%. The residue crystallized from 50% alcohol in needles. *m.p.* 197–197.5°. $[\alpha]_D - 10^\circ$ in chloroform. (Found: *N*, 3.1%. $C_{21}H_{27}O_6N$ requires *N*, 3.2%.)

N-Propionyl β -phenylglucosaminide. *N*-Propionyl triacetyl- β -phenylglucosaminide (0.56 g.) was dissolved in methyl alcohol (10 ml.), and *N* sodium methoxide (3 ml.) was added. The solution was left at room temperature for 2 hr., and exactly neutralized with 2*N* H_2SO_4 . The solution was filtered and the sodium sulphate was extracted several times with boiling alcohol. The combined extracts and filtrate were evaporated to dryness, re-extracted five times with hot alcohol and evaporated to a low volume from which the compound crystallized in needles. Yield 0.38 g. (95%). *m.p.* 230° with decomp. $[\alpha]_D + 8^\circ$ in pyridine. (Found: *C*, 57.73; *H*, 6.78; *N*, 4.44%. $C_{13}H_{21}O_6N$ requires *C*, 57.9; *H*, 6.75; *N*, 4.5%.)

N-Butyryl triacetyl- β -phenylglucosaminide. This compound was prepared from triacetyl- β -phenylglucosaminide and butyryl chloride in the same way as the propionyl compound. Yield 0.33 g. The substance crystallized from aqueous alcohol in needles. *m.p.* 178–179°. $[\alpha]_D - 10^\circ$ in chloroform. (Found: *N*, 3.1%. $C_{22}H_{29}O_6N$ requires *N*, 3.1%.)

(2) Enzyme experiments

Preparation of the enzyme solution from Helix pomatia. Fifteen snails were dissected, and the whole of the digestive tract and liver were removed. The organs were ground with clean sand and extracted with 100 ml. *a.r.* glycerol. The glycerol extract was filtered through muslin, and the sand and macerate were re-extracted twice with 25 ml. glycerol and filtered. Solid matter was removed by centrifuging, and the glycerol extract was dialysed for 16 hr. against running tap water and for 2 hr. against distilled water. It was necessary to use a bladder for dialysis, since the cellulase in the extract destroyed cellophane membranes. The aqueous extract (250 ml.) after dialysis was kept at 2°, and retained its activity with respect to β -glucosidase and glucosaminidase after 4 months. The cellulase activity disappeared in time. When the solution was first prepared it was opaque, and no solid matter could be removed by centrifuging, but it cleared over a period of 3 months, a light brown precipitate settling in the bottom of the flask, and eventually became translucent.

Methods of determining enzyme activity. Two methods were used. For methylglucosaminides and polysaccharides, the reducing sugar liberated by hydrolysis of the glycoside link was estimated by Hanes' [1929] modification of the method of Hagedorn & Jensen. It was found by Mogridge & Neuberger [1938] that the amount of thiosulphate needed was strictly proportional to the amount of *N*-acetylglucosamine present, 2.48 ml. of *N*/100 thiosulphate being equivalent to

1 mg. of *N*-acetylglucosamine. This method was not applicable in the case of phenylglucosaminides, since the phenol liberated also takes up iodine but not in stoichiometric proportions. For these experiments, phenol determinations by the method of Folin & Ciocalteu [1927] were done. β -Glucosidase activity was measured by the rate of hydrolysis of salicin, the saligenin liberated being measured by the method of Folin & Ciocalteu [1927].

The samples to be estimated were treated as follows: 2 ml. were removed from the hydrolysis solution and 3 drops of 5% sodium bicarbonate solution and 1 ml. of dialysed iron (the stock solution B.P. was diluted ten times) were added. The solution was filtered and washed with water till the volume was 10 ml. An aliquot was taken and estimated by one of the methods given.

Effect of pH on N-acetylglucosaminidase. The pH range from 1.5 to 9.0 was covered by buffers made up as follows: the lowest points were obtained by adding suitable amounts of HCl to the reaction mixture; the range from pH 3 to 6 was covered by phthalate and acetate buffers. This was done in order to determine whether alteration in the anion produced any effect on the activity of the enzyme. The higher pH range was covered by phosphate and borate buffers. Tubes containing 10 mg. of β -phenyl *N*-acetylglucosaminide, 0.3 ml. of enzyme solution, 0.2 ml. of buffer and 1.5 ml. water were left for 5 hr. at 38°, and the amount of phenol liberated was determined colorimetrically by the method of Folin & Ciocalteu [1927]. Control experiments were done on the substrate and buffer from pH 4 downwards, since it was expected that acid hydrolysis of the glucosaminide might be significant; actually, however, even at pH 2.5 less than 0.5% of phenol was liberated by acid hydrolysis. The true pH was measured, using a capillator, since it was found that the enzyme solution itself exerts a buffering action. The results are given in Table I.

Table I

	pH	% splitting
HCl	2.6	25.2
	3.2	66.0
Phthalate	3.3	68.0
	3.6	71.0
	4.0	65.0
	4.5	56.0
	5.0	43.0
	5.5	35.6
	6.0	27.6
Acetate	4.0	63.6
	4.5	59.8
	5.0	45.3
	5.5	34.1
Phosphate	6.5	23.4
	7.0	21.7
Borate	9.0	9.9

It appears, therefore, that within the limits of experimental error, the anion of the buffer has no effect on the enzyme. The pH optimum for the β -glucosaminidase is 3.6.

Kinetics of the enzymic hydrolysis. For purposes of standardization, it was considered important to investigate the time relationship of enzymic hydrolysis. 50 mg. of β -phenyl *N*-acetylglucosamine were dissolved in 15 ml. of water; 2.5 ml. of phthalate buffer, pH 4.0, and 2.5 ml. of enzyme solution were added. The reaction mixture was kept at 38°, and 2 ml. portions were removed at short intervals. The results are given in Table II. *k* is calculated as a constant for a unimolecular reaction.

Table II

Time (min.)	% splitting	<i>k</i>
20	21.8	0.00123
40	34.2	0.00104
60	46.3	0.00103
80	57.2	0.00106
100	64.6	0.001035
120	70.0	0.00100
160	79.6	0.00099
200	82.2	0.00086
270	83.8	0.00067

From Table II it appears that, up to about 65% hydrolysis, the reaction follows a unimolecular course. Afterwards, there is an increasing diminution of *k*, a phenomenon which has been often observed in enzymic reactions.

By extrapolation of the data given in Table II, it was found that the half-time value for this concentration of enzyme and substrate was 75 min. This experiment was repeated under almost identical conditions after an interval of 12 weeks, and the half-time value was found to be the same.

Effect of temperature on the Helix pomatia glucosaminidase. Samples containing 5 mg. of β -phenyl *N*-acetylglucosaminide, dissolved in 1.5 ml. water, with 0.25 ml. phthalate buffer (pH 4.0) and 0.25 ml. enzyme solution, were left at temperatures of 0, 9, 17.5, 25, 37 and 54.4°, and the amount of hydrolysis after 1.5 hr. was determined.

Table III

Temperature (°C.)	% splitting
0	13.1
9	23.6
17.5	39.3
25	54.7
37.5	61.7
54.5	39.3

It appears that the optimum temperature for this enzyme is at 37.5°. The activity of the enzyme is completely destroyed if the solution is boiled for 30 sec.

Action of Helix pomatia glucosaminidase on N-acetylmethylglucosaminides. Small test tubes containing 4 mg. of *N*-acetyl methylglucosaminide, 0.1 ml. of *M*/10 phthalate buffer (pH 3.5) and 0.1 ml. of enzyme solution were made up to 2 ml. with distilled water and 2 drops of toluene were added. The tubes were removed at measured intervals of time, the amount of splitting being measured by Hanes' method. Measurement at zero time showed that the enzyme solution has an initial reducing value, but a control experiment carried out at 38° on the enzyme solution showed that this value did not alter with time. The percentage hydrolyses of α - and β -methyl *N*-acetylglucosaminides are given in Table IV.

Table IV

Time (hr.)	% splitting	
	β -Methyl <i>N</i> -acetylglucosaminide	α -Methyl <i>N</i> -acetylglucosaminide
3	6	0
6	10	0
24	35	0
48	56	0
65	—	0

It appears, therefore, that α -methyl *N*-acetylglucosaminide is not split by *Helix pomatia* enzyme under the conditions of the above experiment.

Action of Helix pomatia enzyme on methylglucosaminide hydrochloride. Samples containing 5 mg. of β -methylglucosaminide hydrochloride, 0.2 ml. of enzyme solution, and 0.2 ml. buffer (pH 4.0) were made up to 2 ml. with distilled water. These were analysed for reducing sugar by Hanes' method at zero time and after 24 hr. No free sugar beyond the initial value was found.

Helix pomatia glucosaminidase therefore does not hydrolyse non-acylated glucosaminides.

Action of Helix pomatia enzyme on O-substituted glucosaminides. *N*-acetyl trimethyl- β -methylglucosaminide [Cutler *et al.* 1937] and *N*-acetyl triacetyl- β -phenylglucosaminide [Helferich & Iloff, 1933] were both tested for enzymic hydrolysis, the former by Hanes' method and the latter by the method of Folin & Ciocalteu. In neither case was any hydrolysis observed.

Action of Helix pomatia enzyme on different N-acyl derivatives. The action of the glucosaminidase on *N*-*p*-toluenesulphonyl- β -phenylglucosaminide was determined under slightly different conditions from the other experiments; owing to the low solubility of the substrate, a higher concentration of enzyme was used. After 24 hr. 0.5% splitting was observed, but this is within the limit of experimental error.

Since this compound was not hydrolysed, it was thought to be of interest to discover whether this result was due to a steric effect owing to the very large size of the *N*-substituent; the action of the enzyme on *N*-propionyl and *N*-butyryl β -phenylglucosaminides was therefore studied. In both cases no measurable hydrolysis occurred.

The action of the enzyme on *N*-formyl β -methylglucosaminide was then determined by Hanes' method, a comparative experiment on the hydrolysis of *N*-acetyl β -methylglucosaminide being done simultaneously. A blank determination on the *N*-formyl compound was first done in order to discover whether the *N*-formyl group was removed under the conditions of Hanes' determination, and if so, whether the sodium formate so formed had a reducing value. No reduction was observed.

Each substrate (0.02 g.) was dissolved in 6 ml. water, and 4 ml. of the enzyme solution and 1 ml. sodium phthalate buffer (pH 4.0) were added; a blank experiment was done on the enzyme solution, and it was found that the initial reducing value of the enzyme does not increase with time. For the calculation of percentage hydrolysis, it has been assumed that the molecular equivalent in terms of thiosulphate is the same for *N*-formyl as for *N*-acetyl glucosamine. The results are given in Table V.

Table V

Time (hr.)	% splitting	
	<i>N</i> -acetyl	<i>N</i> -formyl
3	23.7	20.1
5	36.4	31.0
6	40.2	34.6
7½	45.0	40.2
21	69.8	70.5

Separation of β -glucosidase and glucosaminidase in Helix pomatia extract. Attempts were made to separate the β -glucosidase from glucosaminidase by filtration through a column of bauxite [Zechmeister *et al.* 1938]. The filtration was done as follows: Hungarian bauxite roughly ground was mixed with 30% of its weight of fine sand, and 18 g. of the mixture were placed in a tube 10 cm. long, diameter 2 cm., until the height in the tube was 5 cm.; the powder was kept in the

tube with glass wool. 10 ml. *N*/50 acetate buffer *pH* 4.7 were added, and sucked through the filter under low pressure. As soon as the level of the buffer had reached the top of the bauxite column, 25 ml. of enzyme solution were added. The first 10 ml. of filtrate were discarded, and the filtered enzyme was then collected. Alterations in enzyme content of the solution were tested as follows: the amount of β -glucosidase present was determined by testing the activity of the solution towards β -phenyl *N*-acetylglucosaminide at *pH* 4.0 and at 38°. The concentrations of substrate, enzyme and buffer were the same in both instances. In two experiments, considerable separation was effected, as can be seen in Tables VI and VII.

Table VI

Time (hr.)	Crude enzyme splitting by		Filtered enzyme splitting by	
	Glucosidase %	Glucosaminidase %	Glucosidase %	Glucosaminidase %
2	14.5	52	0.5	30
4½	23.3	50	0.9	58

Table VII

Time (hr.)	Crude enzyme splitting by		Filtered enzyme splitting by	
	Glucosidase %	Glucosaminidase %	Glucosidase %	Glucosaminidase %
14	100	90	20	65

Several unsuccessful attempts were made to repeat this separation, using bauxite from an unknown source, and also Hungarian bauxite. It appears that very small unknown changes in the conditions of the filtration either allow both enzymes to come through equally, or cause both to be retained on the absorbent.

Effect of the crude and filtered enzyme on chitin. The crude enzyme was found to hydrolyse chitin slowly, 22 % hydrolysis being found after 254 hr. The action of the filtered enzyme could not be determined, since it was too unstable to retain its activity for a time sufficient to test on chitin whose enzymic hydrolysis is very slow.

DISCUSSION

It has been found, in studying the hydrolysis of *N*-acetyl β -phenylglucosaminide by *Helix pomatia* enzyme, that up to about 65 % hydrolysis, the reaction is approximately unimolecular. The behaviour of the β -glucosidase contained in emulsin towards several substrates follows a similar course. Later slowing of the reaction can easily be explained by the assumption that slow destruction of the enzyme occurs with increasing temperature; this is also suggested by a decrease in the value of the apparent activation energy of the reaction at temperatures above 25°.

Table VIII

Temperature °C.	% splitting	$\frac{k_t}{k_{t_0}}$	Apparent activation energy (cal.)
0	13.1	1.00	$E_{0 \rightarrow 9} = 11020$
9	23.6	1.92	$E_{9 \rightarrow 17.5} = 11540$
17.5	39.3	3.55	$E_{17.5 \rightarrow 25} = 10620$
25	54.7	5.64	$E_{25 \rightarrow 37.5} = 2830$
37.5	61.7	6.83	
54.5	39.3	3.55	

By extrapolation of the data given in Table III, the temperature optimum appears to be at 37.5°, although it may be slightly lower. In any case, the

optimum is not sharp: changes in temperature between 28 and 38° do not greatly alter the amount of hydrolysis.

It appears from Table VIII that $E_{37.5 \rightarrow 54.5}$ will have a negative value. The apparent energy of activation is reasonably constant between 0 and 25°, but falls off enormously between 25 and 38°. The negative value obtained for E at temperatures higher than 30° indicates that the temperature increment of inactivation of the enzyme is much larger than that of the reaction. Actually, the expression energy of activation has no strict physical meaning for enzyme reactions, but it has been used in this instance as a convenient method of determining the temperature increment of the reaction in the range 0 to 25°, where it remains reasonably constant.

The pH optimum for *Helix pomatia* glucosaminidase is 3.6, which is lower than that of any other carbohydrase, and is also considerably lower than the optimum, at pH 5.2, which Karrer & Hofmann found for the hydrolysis of chitin by chitinase in *Helix pomatia*. This, however, does not necessarily indicate that chitinase and β -glucosaminidase are different enzymes, since several instances are known in which the substrate may exert a marked effect on the pH optimum.

Specificity of the Helix pomatia enzyme

The failure of the enzyme to hydrolyse *N*-acetyl α -methylglucosaminide, together with Zechmeister's¹ observation that the same enzyme will not hydrolyse *N*-acetyl α -phenylglucosaminide, shows definitely that it is active only towards β -compounds. Further, a substrate containing a free amino group is not hydrolysed by the enzyme; the same behaviour was also observed by Helferich *et al.* [1934] in the glucosaminidase of emulsin, which will not attack a free phenylglucosaminide.

In order to enable enzymic hydrolysis to proceed, however, it is not sufficient to acylate glucosaminides in a random manner. It is hardly surprising that the enzyme is unable to hydrolyse a glucosaminide which is acylated with an artificial aromatic group like *N*-*p*-toluenesulphonyl; more remarkable is its inability to hydrolyse glucosaminides acylated with propionyl and butyryl groups, which are closely related to the acetyl group. The fact that *N*-formyl β -methylglucosaminide is hydrolysed by the enzyme, at a rate comparable with that of the corresponding *N*-acetyl compound, is of considerable interest in view of Miles & Pirie's observation [1939] that the *N*-formyl group occurs naturally in bacterial antigens, and it is tempting to correlate these facts, namely, that the only acylated glucosaminides hydrolysed by the enzyme are the *N*-formyl and *N*-acetyl derivatives, and that these acyl groups are the only *N*-acyl substituents so far known to occur in nature.

This specificity towards different *N*-acyl substituents may be explained by a steric hindrance of the hydrocarbon chain in *N*-propionyl and *N*-butyryl glucosaminides. It is nevertheless surprising that lengthening of the chain by one methylene group should not only slow down but completely prevent enzymic hydrolysis.

Blocking of the hydroxyl groups on carbon atoms 3, 4 and 6 by methylation or acetylation also prevents enzymic hydrolysis. This seems to indicate that the enzyme requires at least one free hydroxyl group to enable it to combine with the substrate.

¹ Zechmeister & Toth have obtained identical results [1939] as to the stereochemical specificity of the enzyme.

It is interesting to note that, as in the case of β -glucosaminidase in emulsin, the *Helix pomatia* enzyme hydrolyses β -methylglucosaminide much more slowly than β -phenylglucosaminide, the ratio being 1 : 34.

We have previously shown [Neuberger & Pitt Rivers, 1939] that α - and β -methylglucosaminides possess *cis* and *trans* configurations respectively. The determination of chemical specificity justifies the description of the *Helix pomatia* enzyme as a *trans*-*N*-acyl glucosaminidase with restricted *N*-acyl specificity. The instability of the chitinase activity in the purified enzyme preparations which is in contrast with the behaviour of the glucosaminidase activity seems to indicate that the two enzymes are separate.

Configuration of chitin

If Fischer's argument that the hydrolysis of cellobiose by emulsin or by the β -glycosidase of yeast provides adequate evidence of its β -configuration is applied to the hydrolysis of chitin, it may be concluded that this polysaccharide has a β -configuration, since the experiments described above together with those of Zechmeister & Toth [1939], show that the *Helix pomatia* enzyme is specific towards β -compounds. The implicit assumption underlying this argument is that the same enzyme effects the hydrolysis of the polysaccharide and all its intermediates; from our experiments, there is no evidence for or against the validity of this assumption, but experiments by Zechmeister & Toth [1939] indicate that the hydrolyses of chitodextrin and chitobiose are effected by different enzymes or enzyme systems.

The β -configuration of chitin therefore cannot be regarded as finally established; nevertheless, evidence of a different kind points in the same direction. Meyer & Pankow [1935] have shown, by comparison of various physical properties, that chitin and cellulose appear to have similar structures, and from more convincing evidence of X-ray determinations they have found that chitin has a periodicity of 2 sugar units and therefore has a *trans* configuration, which, in the glucosamine series, has been shown by us to correspond to a β -configuration. Further evidence on this point may be found from consideration of the rotation of glucosamine penta-acetates and the acetates of chitobiose and chitotriose. The $[\alpha]_D$ values of α - and β -glucosamine penta-acetates are respectively $+106^\circ$ and $+11^\circ$ in glacial acetic acid (our observations), and the corresponding molecular rotations are $+41,230$ and $+4280$. Chitobiose octa-acetate has been prepared independently by Bergmann *et al.* [1931] and by Zechmeister & Toth [1931], and chitotriose undeca-acetate by the latter authors [1932]. For chitobiose octa-acetate [Zechmeister & Toth, 1931] $[\alpha]_D$ is $+55.3^\circ$ in glacial acetic acid, which gives a molecular rotation of 42,030. From the method of preparation of Bergmann *et al.*, it may be assumed that the compound is an α -acetate, and it can be argued, by analogy with cellobiose which gives the α -octa-acetate on acetylation, that the rotation due to the glucosamine residue carrying the α -acetate will be approximately the same as that of α -glucosamine penta-acetate. The residual rotation due to the other half of the molecule involved in the biose linkage will be the difference between the value for the whole molecule and that of α -glucosamine penta-acetate, i.e. $+800$. This low value strongly indicates a β -configuration and therefore a β -linkage.

If the same calculation is applied to chitotriose undeca-acetate, with $[\alpha]_D + 33^\circ$ in glacial acetic acid [Zechmeister & Toth, 1932], the molecular rotation for the whole molecule is 31,800, and therefore the molecular rotation for each of the two glucosamine residues in which C_1 is involved in the polysaccharide link becomes

$-9400 \div 2 = -4700$. This again suggests a β -configuration for these two linked glucosamine residues.

It might be argued that chitobiose octa-acetate is a β -acetate with an α -biase link, but if this were the case chitobiose undeca-acetate would have two α -linkages and one β -acetate group, which would make the molecular rotation very much higher (probably over 70,000).

For the purpose of this argument, it has been assumed that acetylation and etherification on C_4 do not greatly alter its contribution to the total rotation. The rules of optical superposition cannot be expected to apply quantitatively, since this alteration on C_4 is not negligible. Nevertheless, the calculations made above lend strong support to the theory that chitobiose, chitotriose and chitin have a β -configuration.

SUMMARY

1. An enzyme extract has been prepared from *Helix pomatia* whose reaction characteristics, pH and temperature optima have been determined.
2. From hydrolysis experiments on α - and β -*N*-acetyl methylglucosaminides, it has been shown that the enzyme is specific to β -compounds.
3. Some new acylated glucosaminides have been prepared; the enzyme has been found to hydrolyse only the *N*-formyl and *N*-acetyl compounds.
4. The enzyme is unable to hydrolyse non-acylated glucosaminides and glucosaminides which are completely *O*-substituted.
5. Separation of the β -glucosidase and β -glucosaminidase activities of *Helix pomatia* extract has been effected.
6. Enzymic experiments and other evidence bearing on the structure of chitin are discussed.

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CXCV. THE INTERACTION BETWEEN PORPHYRINS AND LIPOID AND PROTEIN MONOLAYERS

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IN many porphyrin-protein combinations, e.g. in the haemoglobins, cytochrome *c* [Keilin, 1925] and horseradish peroxidase [Keilin & Mann, 1937], there exists a stoichiometric relation between the porphyrin and the protein, indicating a direct chemical combination, which in the case of cytochrome *c* is effected through a thioether linkage in those side chains which in the free porphyrins contain the vinyl groups [Theorell, 1938].

On the other hand, phenomena such as photosensitization, action on paramoecia or transport in blood serum must be attributed to the ability of the porphyrins to form "complexes" or "adsorption" compounds with lipins and proteins. The monolayer technique permits us to examine in some detail the factors which are operative in the formation of such compounds, such as identification of the polar groups involved and the effect of changes in the structure of the non-polar or hydrophobic portions of the molecule or the van der Waals forces of interaction, to which in great measure the stability of the compounds formed is due. A further point of interest is that in extended monolayers of proteins the operation termed denaturation has taken place and whilst the polar groups of the side chains are immersed in the substrate and therefore accessible to reaction, their relative disposition to one another and to those in the neighbouring molecules differs from that in the globular native proteins and this may affect their reactivity.

The solubility of the different porphyrins depends on the number and nature of the polar groups attached to the porphyrin skeleton. In some cases the solubility is sufficiently low for monolayers to be obtainable. The properties of such monolayers have been recently examined by Alexander [1937]. In this investigation their reactions with monolayers of materials of biological importance are described with the object of obtaining a comparison of their behaviour with the same system *in vivo*.

EXPERIMENTAL

The monolayers were spread on the surface of various substrates in a Langmuir trough from suitable solvents, or in the case of proteins, both from solution [Gorter & Grendel, 1926; Mitchell, 1937] or from the solid [Hughes & Rideal, 1932]. The surface pressures of the monolayers *F*, surface tensions of the solutions γ and phase boundary potentials ΔV were measured in the usual manner. The porphyrins were dissolved as sodium salts in weak alkali (0.01–0.001*N*) and only freshly prepared solutions were employed. The reacting substance was injected under the monolayer and mixed with the substrate [Schulman & Rideal, 1937] and any penetration or adsorption followed by noting the changes in pressure and potential at constant area. Since many of the substances injected were capillary active it was necessary to determine the change in surface

* Rockefeller Foundation Fellow, 1938–9.

tension ($\Delta\gamma$) of the solution behind the floating barrier of the Langmuir trough so that the change in pressure on penetration could be evaluated from the expression

$$F = F_{\text{obs}} + \Delta\gamma.$$

In general the monolayers were compressed to an initial pressure of $F = 6$ dynes/cm. and the reactions were carried out at room temperature (17 – 23°).

If a small amount of a not too soluble porphyrin be injected into a solution, it will gradually accumulate at the surface passing through a monolayer stage and eventually build a solid layer several molecules thick, with a pressure increase in some cases of more than 20 dynes/cm. which is far higher than the pressure at which the corresponding monolayer collapses. Sobotka [1937, 1] considers such layers to be multilayers with definite structure, but there seems to be no reason to assume that they represent anything but a surface crystallization, the tendency of the flat rings to associate being very strong. Indeed, all other multilayers examined always reveal the presence of crystals [cf., e.g., Stenhagen, 1938, 1]. The rate of formation of these surface-skins of various porphyrins is shown in Fig. 1. That diffusion plays a large part in governing the rate is shown

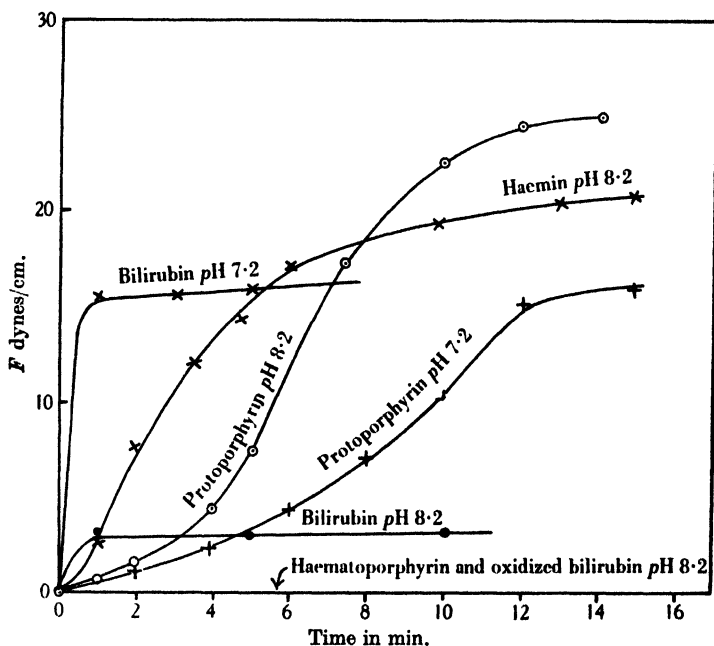


Fig. 1. Surface skin formation of different porphyrins when injected in concentration of $5 \mu\text{g./ml.}$ under a clean water surface $M/25$ phosphate buffers.

by the slow rate of building up by the colloidal solution of protoporphyrin in contrast to the rapid rate revealed by the more disperse bilirubin. Both the meso derivatives in which the vinyl groups have been replaced by ethyl groups and the porphyrin esters are too insoluble to be used for this purpose. In Fig. 2 are shown the results of injection of various porphyrins in concentrations of $5 \mu\text{g./ml.}$ under a cholesterol monolayer at 6 dynes/cm. spread on $M/25$ phosphate buffers at pH 7.2 and 8.2 respectively. Bilirubin, which in the absence of

the cholesterol monolayer, forms a solid skin, which exerts a pressure of $F = 15$ dynes at $pH\ 7.2$ and 3 dynes at $pH\ 8.2$, caused a rapid rise in pressure to 30 dynes and a fall in surface potential of 70 mV. of a cholesterol monolayer at the former pH . The values of 13 dynes and 30 mV. being obtained at $pH\ 8.2$. The monolayer remains liquid in both cases.

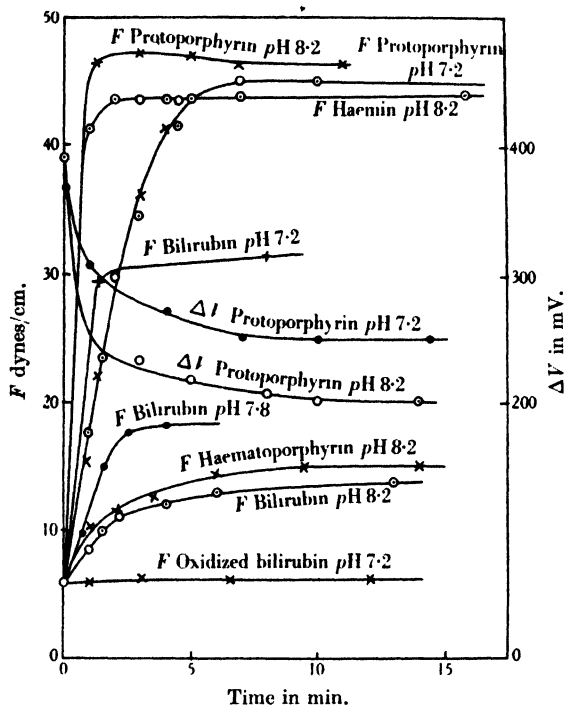


Fig. 2. Effect of pH on penetration into cholesterol on phosphate buffers.

In alkaline solutions, especially when exposed to light, bilirubin undergoes rapid autoxidation. The oxidation product is not capillary active and is not adsorbed by, nor does it penetrate, monolayers of cholesterol. It will be seen that both protoporphyrin and haemin penetrate films of cholesterol quite strongly, the former penetrating more rapidly at $pH\ 8.2$ than at $pH\ 7.2$, this being similar to its behaviour in accumulation at a free surface (Fig. 1). Haematoporphyrin and coproporphyrin, which in the physiological pH range exhibit but little capillary activity, show but a small penetration of cholesterol monolayers, and scarcely any change in surface potential.

Bilirubin in a concentration of $5\mu g./ml.$ injected under monolayers of cetyl alcohol, stearic acid, stearamide or methyl stearate penetrates these films in the same way as it does monolayers of cholesterol, the penetration being stronger at $pH\ 7.2$ than at $pH\ 8.2$. The ester shows the smallest rise in pressure, 23 dynes at $pH\ 7.2$, and a change in surface potential of 160 mV. at $pH\ 7.2$ and 20 mV. at $pH\ 8.2$. The potentials of the cetyl alcohol and the stearic acid monolayer remain practically unchanged and the state of the monolayers remains unaltered, corresponding to that of the original monolayer at the corresponding pressure. Cetyl alcohol is penetrated rather slowly by protoporphyrin, giving a pressure of 20 dynes after 30 min., with a very small change in potential. With octadecylamine

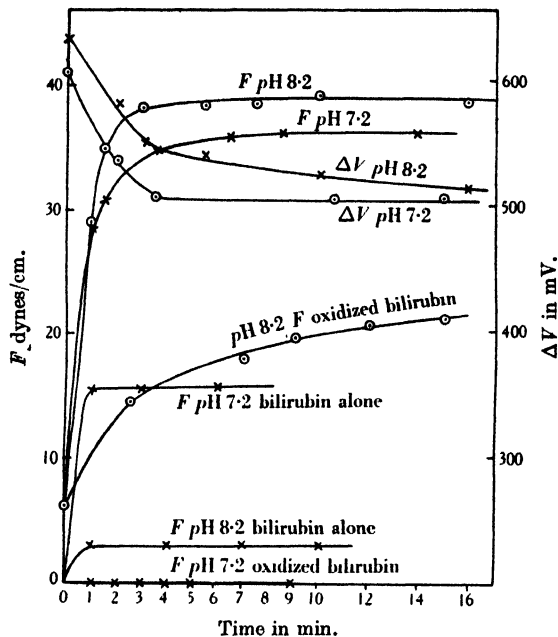


Fig. 3. Penetration of bilirubin into octadecylamine monolayers.

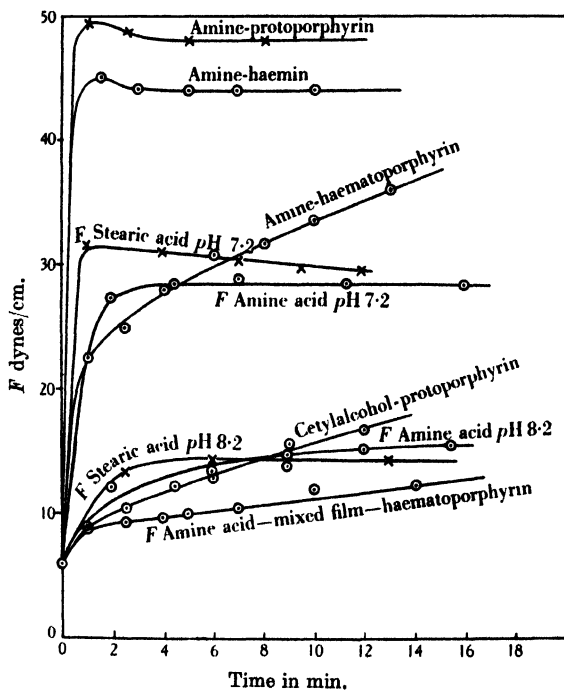


Fig. 4. Penetration of porphyrins into amine and acid monolayers.

monolayers, on the other hand, the behaviour is entirely different and the penetration by bilirubin is in fact somewhat stronger at the more alkaline reaction in spite of the reduced capillary activity (Fig. 3). The potential of the amine monolayer is reduced some 100 mV. The oxidized bilirubin shows a comparatively weak penetration.

Protoporphyrin and haemin also show very strong penetrations of amine monolayers, giving stable pressures of 44–48 dynes (Fig. 4), and reductions in potential of 400 and 200 mV. respectively. The haematoporphyrin penetrates more slowly; the pressure rises to 40 dynes after 20 min., but no change in the potential occurs. Coproporphyrin I penetrates less than haematoporphyrin, the penetration being very weak at pH 8 and increasing with decrease in pH down to 5.5 (20 dynes after 15 min.) after which it decreases owing to complete insolubility of the haematoporphyrin.

The 2,4-dimethyl-5-carbethoxypyrrole-3-acrylic acid was found to penetrate an amine film slowly. If the amine is spread at an area of 50 sq. Å. per molecule on a phosphate buffer pH 6.0 (non-coherent film) and 30 µg/ml. of the acrylic acid be injected, it is found that after 15 hr. the amine is converted into a weak solid, uniform film, exerting a pressure of 6 dynes, probably due to penetration and subsequent surface polymerization of the acrylic acid.

In mixed octadecylamine-stearic acid monolayers, strong polar interaction occurs, as shown by Marsden & Schulman [1938]. If bilirubin is injected under such a monolayer, it is found that the penetration at pH 7.2 and 8.2 is reduced to the same value as obtained with the stearic acid alone and there is no change in the surface potential of the mixed film. Similar results are observed with haematoporphyrin.

Protein monolayers

Bilirubin in a concentration of 5 µg./ml. rapidly increases the pressure of a gliadin monolayer at pH 7.2 to 20 dynes. Identical results are obtained with and without 0.17M NaCl present. The monolayer becomes rigid and behaves like a "tanned" skin, which, however, is not uniform as revealed by the non-uniformity of the surface potential which decreases. At pH 8.2 no tanning occurs, the monolayers remain liquid and there is only a slight increase in pressure (Fig. 5), and no change in the surface potential. Injection of protoporphyrin at pH 7.2 causes only a slow rise in pressure, a pressure of 20 dynes being obtained only after 2 hr. with a slight change in surface potential. At pH 8.2 the "tanning" takes place more rapidly and there is a fall in the surface potential. With haemin at pH 7.2 and 8.2 the behaviour is like that of the protoporphyrin at pH 8.2, but the surface potential increases by 100 mV. This is in accord with the fact that the surface potential of haemin monolayers is higher than that of protoporphyrin [Alexander, 1937].

The tanned films obtained are rapidly dispersed by soaps, in contrast to protein films tanned by tannic acid [Schulman & Rideal, 1937]. Haematoporphyrin shows only a weak adsorption to gliadin films at 6 dynes pressure, and with coproporphyrin and turacin (copper-uroporphyrin) no interaction is observed.

Serum albumin and other protein monolayers are interacted with by injections of porphyrins much in the same manner as gliadin, and no specificity in the different proteins was observed. The behaviour of the porphyrins with proteins was examined further by carrying out some electrophoresis experiments with electrophoretically isolated human serum albumin and different porphyrins, using the micro apparatus of Tiselius [1938]. The isoelectric point of

human serum albumin at 0° is 4.64 [Stenhagen, 1938, 2]. 0.5% protein solution was used. In the place of the photographic plate, a spectrometer with a long vertical slit was employed, thus permitting a direct examination of the absorption spectrum of the solution in different parts of the electrophoresis U-tube to

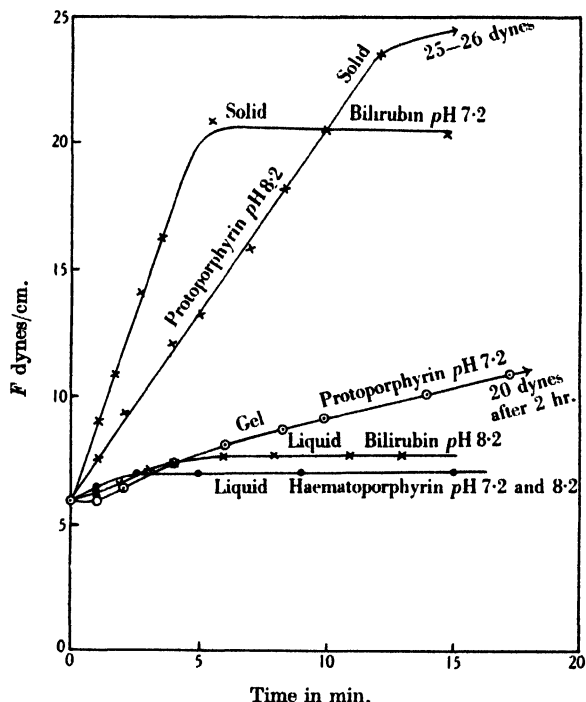


Fig. 5. Gliadin monolayers on $M/25$ phosphate buffers; porphyrins in concentration $5 \mu\text{g./ml.}$ injected.

be made. The Toepler "Schlieren" bands appeared as dark horizontal lines. In a phosphate buffer at $\text{pH } 7.0$, $\mu = 0.1$, it was found that bilirubin in a concentration of $80 \mu\text{g./ml.}$ was entirely absorbed on the protein [cf. studies by Bennhold, 1932], only one boundary on each side being observed. At $\text{pH } 8.2$, however, part of the bilirubin was found to migrate in front of the serum albumin. At $\text{pH } 7.0$, the migrating boundaries were markedly convex in the direction of migration. This indicates an increase in viscosity of the protein solution, as a similar phenomenon occurs with the very viscous solutions of thymonucleic acid [Theorell & Stenhagen, 1939]. Protoporphyrin in concentration $50 \mu\text{g./ml.}$ is entirely absorbed by a 0.5% serum albumin solution, there being no sign of free migrating porphyrin, but haematoporphyrin under the same conditions is only partially adsorbed, free porphyrin being found to migrate in front of the protein. In no case was more than one protein boundary on each side observed, and the free porphyrin boundary was very diffuse.

Hill & Holden [1926] concluded from absorption spectrum analysis that protoporphyrin and globin combine in neutral solution and Haurowitz & Waelsch [1929] observed the same for haematoporphyrin and horse serum. Holden [1937] reinvestigated the problem and concluded from examination of the ultraviolet absorption spectra that protoporphyrin and haematoporphyrin

can combine with horse serum. The electrophoresis experiments support this but show that the haematoporphyrin-serum albumin compound is weaker than the protoporphyrin one. Thus we note a parallelism between the absorption as observed in bulk and the adsorption to protein films, in spite of the different states of the protein which suggests that forces of the same type are operative.

DISCUSSION

It seems clear that, with the exception of the amine group, there is only a weak interaction between groups such as $-\text{OH}$, $-\text{COOH}$, $-\text{COOCH}_3$, $-\text{CONH}_2$ and the reactive groups in the porphyrins, and that for monolayers containing these groups the hydrophobic portions of the reacting molecules play a governing part in complex formation as the stability increases with decreasing water solubility of the penetrating molecule. The marked differences with small changes in pH observed with bilirubin may have a biological significance.

Bennhold [1932] showed that bilirubin in blood is absorbed by the albumin fraction of the serum protein, and Pedersen & Waldenström [1937] showed by means of electrophoretic and ultracentrifugal studies that this absorption is specific for serum albumin and that it does not occur with egg albumin. They attributed this difference to the different amino-acid distributions in the two proteins. From the behaviour of bilirubin towards amine films, we may conclude that the coupling takes place via the primary amino groups in the proteins and the phenolic group in the bilirubin. This is further supported by the fact that in the presence of protein involving a change of the $-\text{OH}$ to a $\text{C}=\text{O}$ group [see Fischer & Orth, 1937] bilirubin is stable against oxidation. The percentage of lysine which has a free amino group in the ϵ -position is much higher (13.2 %) in serum albumin than in egg albumin (3.8 %) and in serum globulin. The actual distribution of the amino groups in the protein molecules with respect to neighbouring acid groups appears to be important, for we have shown that if the amino groups are allowed to interact with acid groups (as in the mixed amine-acid films) the reactivity of the amine groups is very much reduced. This factor, together with the relatively small proportion of amino groups present, can be advanced in explanation of why there is no apparent specificity in the adsorption of bilirubin by protein monolayers. The process of spreading a protein involves an unfolding and reorientating of the protein molecules, and bringing chains of different molecules in close proximity to one another, which upsets the original distribution of polar groups. We have also not been able to reproduce the adsorption experiments by Grollman [1925] with phenol red and serum and egg albumin with monolayers of the proteins.¹

The experiments show that the bilirubin-amine interaction is stronger at pH 8.2 than at pH 7.2. Pedersen & Waldenström [1937] have shown that in the bile the bilirubin is adsorbed on to a colloidal carrier of unknown nature, which increases the solubility of the bilirubin. If the bilirubin-carrier coupling is dependent on interaction with amine groups in the carrier, a decrease in pH will tend to weaken this interaction, and at the same time decrease the solubility of the bilirubin and increase its complex formation tendency with cholesterol. In the complex process of the formation of gall stones of the mixed type, containing bilirubin-calcium-cholesterol-protein, we might therefore expect that a decrease in pH will increase the rate of stone formation. In many infections of the gall

¹ On the other hand selectivity has been observed when the various native proteins are injected under monolayers of certain lipoids in some recent experiments by Schulman & Rideal.

bladder the pH is decreased and some evidence in the literature shows [Sobotka, 1937, 2] that gall-stone formation tendency is increased with decrease of the pH in the gall bladder.

SUMMARY

It is shown that bilirubin and the capillary-active porphyrins strongly penetrate lipid monolayers and that this penetration involves but a weak polar interaction and is largely influenced by the interaction of the hydrophobic portions of the molecules. Specific interaction is found, however, to occur with amine monolayers, the strength of the interaction being independent of the capillary activity of the reactant. Protein films interact with and are tanned by the more capillary active porphyrins, but in some cases no adsorption of the porphyrins occurs in spite of strong reaction with amine films. It is shown that this is due at least in part to polar interaction within the protein monolayer itself, as the reactivity of the amine group is negligible in mixed amine-acid films, where strong polar attraction between the basic and the acid groups occurs. Some electrophoresis experiments in porphyrin-serum albumin mixtures in bulk solution are described and compared with the data obtained in monolayers. Possible biological implications of the results are discussed.

We are indebted to Prof. F. Haurowitz, Prague, Prof. H. Fischer, Munich, Dr W. Siedel, Munich, Prof. Keilin, Cambridge, and Dr Jan Waldenström, Uppsala, for gifts of the various porphyrins used in the investigations. The electrophoresis experiments were carried out at the Institute of Physical Chemistry, Uppsala. We are indebted to Prof. A. Tiselius for permitting his apparatus to be used and to Miss G. Gustbée for assistance in the experiments.

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CXCVI. STUDIES OF THE PHYSICO-CHEMICAL STATE OF CALCIUM IN THE SERUM OF THE RUMINANT

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ALTHOUGH it is now well established that all the Ca of serum is not in the same physico-chemical state, unanimity does not yet exist regarding the nature of the different fractions. All workers are agreed that a primary separation into two forms, ultrafiltrable and non-ultrafiltrable, can be made but disagreement arises as to whether these two fractions can be individually subdivided into other forms. McLean & Hastings [1934; 1935] hold that the ultrafiltrable Ca is entirely ionic (with the exception of a trace bound by citrate) and that this ionic fraction is in equilibrium with the non-ultrafiltrable Ca which they consider to be entirely Ca proteinate. Benjamin [1933] presented the view that the ultrafiltrable Ca exists in two forms, the one ionic and the other a complex ion consisting of Ca, phosphate and, probably, bicarbonate ions. She further regarded the non-ultrafiltrable fraction as being divided into two forms but did not present any studies of the chemical nature of the fractions although it was presumed that one of them was in some way bound by serum proteins. Although the use of adsorbents for the fractionation of serum Ca had been attempted by various workers, Benjamin & Hess [1933] were the first to standardize the preparation of the adsorbent (BaSO_4) and to establish the conditions under which maximal and reproducible adsorptions occurred in serum samples.

Duckworth & Godden [1936] studied the changes which occur in the physico-chemical state of serum Ca when citrate ions are introduced into the system. The study involved intrajugular injections into a cow and the examination of serial samples of serum for the distribution of Ca in its various fractions. Similar studies were performed *in vitro*. The changes observed were found to support the view of Benjamin & Hess. Further, data obtained in studies of the fractionation of serum Ca in sheep were calculated according to the McLean-Hastings expression

$$\frac{[\text{Ca}^{++}][\text{Prot.}^-]}{[\text{Ca proteinate}]} = K$$

(concentrations in mols. at 25° and $\text{pH } 7.35$). The value found for K by McLean & Hastings was $10^{-2.22 \pm \text{s.d. } 0.07}$ for their data and Duckworth & Godden obtained a value of $10^{-2.10 \pm \text{s.d. } 0.08}$ in their experiments. The latter authors pointed out that similar ranges were obtained in both cases, but that such a high degree of variability was evidence of factors within the system influencing the distribution of Ca in its different forms, but not considered in the expression. This variability could not have been caused by the method of measurement alone since, although McLean & Hastings used a biological method of measurement, Duckworth & Godden used only chemical methods.

The BaSO₄-adsorption technique of Benjamin & Hess has been criticized by Greenberg & Larson [1935] but, since Benjamin [1935] has published a reply, no further comment need be included at the present time.

The primary purpose of the present research was to study the influence of different dietary contents of Ca and phosphorus on serum Ca and inorganic P in sheep and, further, to investigate the variations produced in the partition of serum Ca by the changed levels of these serum constituents. Similar studies were made of the effect of fasting on these constituents in cows, sheep and goats. In addition, an opportunity presented itself in the investigation for a study of the McLean-Hastings dissociation constant under conditions of fluctuating serum inorganic P values in the same animal. This was felt to be important since McLean & Hastings consider that any influence of serum inorganic P on the partition of serum Ca can be ruled out while Benjamin & Hess consider that the inorganic P of serum is, in part, a component of one of the Ca fractions and should, therefore, influence the distribution. For this reason serum proteins were also determined.

METHODS

In the case of sheep and goat blood samples were drawn from the jugular vein while employing partial stasis. In the experiments with cows mammary venous blood was obtained. The serum was separated as rapidly as possible.

The following analytical methods were used:

Serum Ca partition. The method of Benjamin & Hess [1933]. The membranes were prepared according to Duckworth [1935]. All Ca estimations were performed according to Clark & Collip [1925].

Serum inorganic P. According to Bodansky [1932-3].

Serum true protein. 6.25 (total N (macro-Kjeldahl) - N.P.N. (micro-Kjeldahl)).

The nomenclature of Duckworth & Godden is used for the different serum Ca fractions:

Ca ⁺⁺	= calcium ion;
U. Ca C.	= ultrafiltrable calcium complex;
N.U. Ca C.	= non-ultrafiltrable calcium complex;
P.B. Ca	= protein-bound calcium;
T. Ca	= total serum calcium.

EXPERIMENTAL

Exp. 1. It was previously shown by Godden & Ray [1938] that when growing sheep were fed a P-deficient ration the serum inorganic P was gradually reduced. In about 4 weeks the average level fell to 2.86 mg. per 100 ml. serum. When a P supplement was added to the ration the serum inorganic P values increased and, at the end of 4 weeks, the average value was 8.41 mg. During these changes the serum Ca varied inversely with the inorganic P, the average serum Ca level being 13.4 mg. at the end of the first period and 9.5 mg. at the end of the second period.

Four sheep were fed and managed as in the above experiment. The investigation was divided into four feeding periods, a description of which is given in Table I. Blood samples were drawn twice during periods 1, 2 and 4, once when the period was well advanced and again at the end of the period. Only one sample was drawn during period 3—at the end of the period. The results are given in Table II and the averages are shown in Fig. 1.

Table I. *Dietary periods and intakes of C and P*

Feeding period 1 (from 29. vii. 37 to 16. ix. 37). Basal ration (low in P)	Daily level of intake (g.)	Ca : P = 8.2 : 1
	$\left. \begin{array}{l} \text{Ca} = 3.84 \\ \text{P} = 0.47 \end{array} \right\}$	
Feeding period 2 (from 17. ix. 37 to 26. x. 37). Basal ration plus KH_2PO_4	Daily level of intake (g.)	Ca : P = 1.6 : 1
	$\left. \begin{array}{l} \text{Ca} = 4.57 \\ \text{P} = 2.80 \end{array} \right\}$	
Feeding period 3 (from 27. x. 37 to 9. xi. 37). Basal ration plus NaH_2PO_4 and CaCO_3	Daily level of intake (g.)	Ca : P = 8.2 : 1
	$\left. \begin{array}{l} \text{Ca} = 22.93 \\ \text{P} = 2.80 \end{array} \right\}$	
Feeding period 4 (from 9. xi. 37 to 15. xii. 37). Basal ration	Daily level of intake (g.)	Ca : P = 9.3 : 1
	$\left. \begin{array}{l} \text{Ca} = 3.33 \\ \text{P} = 0.36 \end{array} \right\}$	

Table II. *Effect of changes in dietary Ca and P intakes on the composition of blood serum*

(Results given per 100 ml. serum.)

Sheep no.	Inorg. P mg.	T. Ca mg.	Ca ⁺⁺ mg.	U. Ca C. mg.	N.U. Ca C. mg.	P.B. Ca mg.	True protein g.	pK
25. vii. 37. Low P ration. Period 1								
5	2.92	12.7	3.6	5.2	0.7	3.2	6.10	1.82
6	3.04	11.8	3.2	4.8	1.2	2.6	6.08	1.84
7	2.76	13.2	3.8	5.2	0.9	3.3	6.20	1.83
8	2.88	12.4	3.8	4.6	1.0	3.0	6.19	1.83
17. ix. 37. Low P ration. Period 1								
5	2.62	12.8	4.0	5.0	0.5	3.3	6.04	1.79
6	2.84	13.0	3.7	5.1	0.9	3.3	6.11	1.85
7	2.48	13.6	4.1	5.3	1.1	3.1	6.09	1.81
8	2.52	12.4	3.6	4.9	0.7	3.2	6.14	1.82
7. x. 37. P supplemented ration. Period 2								
5	6.74	10.4	1.2	4.7	2.2	2.3	6.11	2.06
6	8.02	9.8	1.1	4.3	3.1	1.3	5.93	2.10
7	5.96	10.6	1.4	5.4	2.1	1.7	6.20	1.90
8	6.28	10.7	1.5	4.7	2.9	1.6	5.91	2.06
26. x. 37. P supplemented ration. Period 2								
5	7.22	9.4	1.1	5.0	2.1	1.2	5.92	1.91
6	8.40	10.3	1.6	4.7	2.2	1.8	5.87	1.98
7	6.04	10.3	1.4	4.8	2.0	2.1	6.00	1.99
8	5.92	10.9	2.0	5.8	1.4	1.7	5.94	1.77
10. xi. 37. P supplemented ration + CaCO_3 . Period 3								
5	3.04	11.6	2.8	4.6	1.7	2.6	5.84	1.94
6	2.98	10.7	2.2	4.2	1.4	2.9	6.02	2.01
7	3.12	12.1	2.9	4.9	1.9	2.4	6.28	1.90
8	3.46	11.0	3.2	3.7	1.2	2.9	5.91	1.96
29. xi. 37. Low P ration. Period 4								
5	2.22	13.6	3.9	5.1	0.9	3.7	6.18	1.88
6	2.84	14.1	4.4	5.0	1.2	3.5	6.29	1.86
7	2.54	14.4	4.3	5.0	1.1	4.0	6.22	1.91
8	2.62	13.2	3.9	4.9	1.0	3.4	6.31	1.86
17. xii. 37. Low P ration. Period 4								
5	2.24	13.2	3.8	4.8	1.1	3.5	6.10	1.90
6	2.76	14.4	4.4	5.4	1.3	3.3	6.32	1.83
7	2.48	13.9	4.3	5.0	1.0	3.6	6.27	1.86
8	2.48	14.0	4.1	5.3	0.8	3.8	6.14	1.86

Exp. 2. Advantage was taken of the availability of blood samples in an experiment being independently conducted for a study of the effects of prolonged fasting in ruminants and the partition of serum Ca was investigated. Altogether

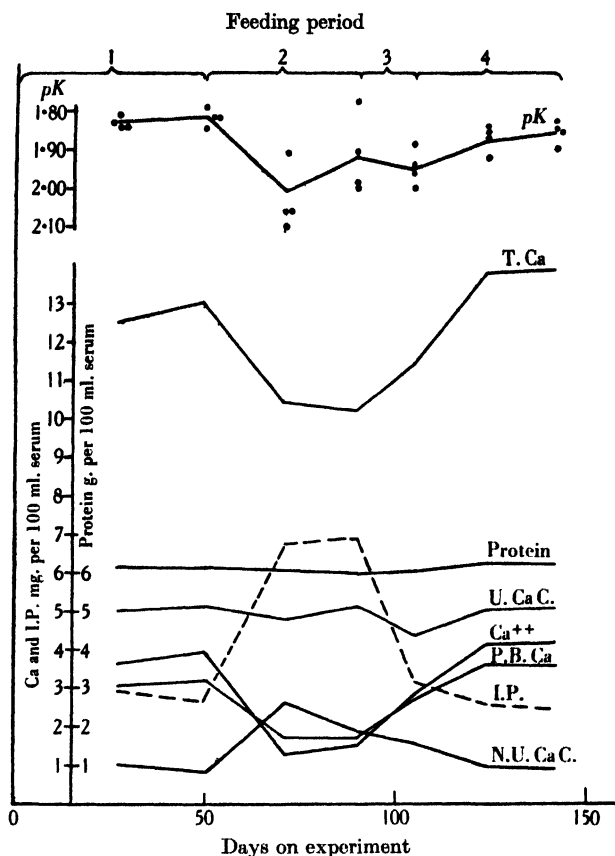


Fig. 1. Effect of diets of various Ca and P contents on blood composition.

11 animals were used—4 growing sheep, 2 non-lactating goats, 2 lactating goats and 3 lactating cows. The goats fasted for 12 days, the sheep for 7 days and cows nos. 1 and 2 for 12 days. Cow no. 3 died on the eighth day of the fast. The results are recorded in Table III.

RESULTS

Exp. 1. The results obtained from altering the Ca and P contents of the diet of the sheep in this experiment were clear-cut and distinctive. During period 1 serum inorganic P was much lower than the level found in normally fed sheep. After the addition of phosphate to the ration, the P sharply increased to about normal levels. In period 3, where an addition of CaCO_3 to the diet fed in period 2 increased the Ca : P ratio to the same value as in period 1, the serum inorganic P was markedly reduced. During period 4 the serum P showed a slight further reduction.

The total serum Ca behaved in an inverse manner compared with the inorganic P. It was remarkable to find that the U. Ca C fraction of the serum

Ca maintained an almost constant level throughout the experiment. In spite of widely varying concentrations of Ca and inorganic P only small changes occurred in the average values for this constituent. The Ca^{++} and P.B. Ca fractions should be considered together since an equilibrium between them is apparent from the experiments on the effect of citrate on the partition of serum Ca [Duckworth & Godden, 1936]. It is quite clear that the fall in total serum Ca occurs at the expense of these two fractions while their somewhat parallel individual fluctuations are in accordance with the view that an equilibrium exists between them. When phosphate was added to the diet, the N.U. Ca C. increased from the period 1 level and gradually declined until the original level was reached in period 4.

Table III. *Effect of starvation on the blood composition of sheep, goats and cows*

(Results given per 100 ml. serum.)

Day of fast	Inorg. P mg.	T. Ca mg.	Ca^{++} mg.	U. Ca C. mg.	N.U. Ca C. mg.	P.B. Ca mg.	True protein g.	pK
Goat 1. Non-lactating								
0	5.36	9.1	1.8	2.8	1.2	3.3	6.00	2.19
10	6.86	8.2	1.1	2.7	2.3	2.1	6.76	2.19
Goat 2. Non-lactating								
0	6.42	8.5	1.1	3.6	0.9	2.9	6.43	2.05
10	12.00	8.0	0.8	3.1	2.4	1.7	7.13	2.11
Goat 3. Lactating								
0	5.46	9.8	1.8	3.5	1.1	3.4	5.90	2.13
12	7.24	8.1	0.8	3.1	2.3	1.9	6.54	2.18
Goat 4. Lactating								
0	4.92	10.4	2.1	4.2	1.0	3.1	6.69	1.95
12	6.88	8.6	1.0	3.5	1.9	2.2	7.27	2.04
Sheep 5								
0	2.87	13.3	3.2	5.0	1.4	3.7	6.02	1.99
7	9.24	9.9	1.3	3.5	3.0	2.1	6.46	2.18
Sheep 6								
0	3.02	13.2	3.3	5.2	0.9	3.8	6.00	1.93
7	10.00	10.7	1.6	4.1	2.8	2.2	6.66	2.08
Sheep 7								
0	2.94	13.7	4.0	5.0	1.1	3.6	6.14	1.90
7	11.02	10.0	1.1	3.8	2.9	2.2	6.46	2.17
Sheep 8								
0	2.82	12.8	3.8	4.8	1.0	3.2	6.26	1.85
7	9.36	10.5	1.4	4.2	2.8	2.1	6.58	2.11
Cow 1								
0	5.80	8.6	1.6	3.1	0.8	3.1	7.89	1.96
3	4.28	5.8	0.5	2.2	0.8	2.3	9.44	2.01
7	7.36	7.9	0.9	3.3	1.4	2.3	8.22	1.96
12	5.38	7.8	1.2	2.8	1.3	2.5	8.21	1.99
Cow 2								
0	5.12	10.6	2.4	4.1	0.9	3.2	7.60	1.85
2	5.02	10.5	2.2	3.9	0.9	3.5	7.93	1.90
9	6.46	10.2	1.6	4.4	1.4	2.8	8.50	1.84
12	7.22	8.1	1.0	3.1	1.9	2.1	8.35	2.00
Cow 3								
0	4.32	10.5	2.3	4.2	0.7	3.3	6.84	1.91
5	6.24	9.4	1.4	3.8	1.4	2.8	8.46	1.90

The average serum true protein level remained practically unchanged throughout the experiment.

The average pK values showed a definite change during the experiment. The results obtained during period 1 were closely similar at both samplings and were very closely grouped. In period 4, where the same diet was fed, close groupings were again found although the average values were numerically somewhat higher than in period 1. During periods 2 and 3 the pK values were less regular, but nevertheless were clearly displaced from the average of periods 1 and 4. The curve is not regular because of the one aberrant value obtained at the end of period 2. With the exception of this value all other values show a displacement during the periods of higher phosphate intake, the change being most marked at the time of change from period 1 to period 2.

Exp. 2. The effect of fasting was mainly to produce an increase in serum inorganic P in sheep, goats and cows. The changes were not proportional to the length of the fast, the individual variations being so great that conclusions cannot be drawn as to the effect of time on the level of serum inorganic P. Also, the response of the sheep and goats was, in general, much greater in this respect than was that of the cows.

The total serum Ca levels were depressed by fasting and, as observed in Exp. 1, the decreases were chiefly caused by reductions in Ca^{++} and P.B. Ca. Unlike the observations in Exp. 1 the U. Ca C decreased in sheep and goats and, while the same tendency existed, the cows gave somewhat erratic results. In all cases the N.U. Ca C increased in fasting, the increases being roughly proportional to the changes in serum inorganic P.

The serum true protein increased in all cases, the increases being slightly more marked in cows than in sheep and goats.

In sheep and goats, with one exception, the pK values increased numerically during the fast. Such changes as were observed in cows were less marked and somewhat irregular.

DISCUSSION

Duckworth & Godden [1936] previously called attention to the unreliability of the pK values derived from the McLean-Hastings mass law equation and the present observations are in agreement with this conclusion. It is immediately apparent in Fig. 1 that the effect of changing the sheep from a low P to an adequate P diet was to cause a uniform displacement of the pK values, a change which was, to a large extent, reversed by removal of the phosphatic supplement from the ration. That this variation in ultrafiltrability should be inverse to the level of serum inorganic P suggests strongly that the equilibrium existing in the serum is influenced by the level of this constituent. Similarly in Exp. 2 where one effect of fasting was to raise serum inorganic P the pK values, in general, were numerically increased. Such displacements of the pK values indicate that the conception of the original expression failed to include factors which markedly influence the distribution of serum Ca in its various fractions and, since the reductions in ultrafiltrability of serum Ca are associated with increases in serum inorganic P, it seems probable that the older views are in closer agreement with the observed facts.

It might be thought that the reductions in serum Ca which occurred concomitantly with the increases in serum inorganic P in both Exps. 1 and 2 were caused by precipitation of Ca phosphate and further, that a portion of colloidal Ca phosphate was in circulation and thus caused an apparent reduction in ultrafiltrability. While it is possible that the former may have occurred, the latter did

not for two reasons: first, only when serum inorganic P is increased to very high levels does it become partially non-ultrafiltrable [Smith, 1933; 1934]; second, should colloidal Ca phosphate be experimentally produced *in vivo*, it seems probable that it would be rapidly removed from circulation. Since the serum inorganic P did not reach very high levels in these investigations and since the animals were sampled after never less than 15 days on the diet, the possibility of colloidal Ca phosphate existing in the serum samples can be excluded.

The main effects of both fasting and the addition of phosphate to a P-deficient ration were an increase in serum inorganic P and a reduction in serum Ca. In both cases reductions occurred in both Ca^{++} and P.B. Ca. In Exp. 1 the U. Ca C remained fairly constant while in Exp. 2 the values for this constituent were somewhat lowered. This suggests that factors, other than Ca and P alone, govern the quantity of this fraction present in serum. Also the N.U. Ca C showed distinctive behaviour, serum inorganic P elevation in both experiments being associated with increases in the amount of this complex. However, since there seems to be no possibility of the inorganic P having been converted into a non-ultrafiltrable form it cannot be argued that this complex bears any direct relation to serum inorganic P.

SUMMARY

The changes occurring in the partition of serum calcium (*a*) on transferring animals from a phosphorus-deficient diet to a phosphorus-adequate diet, and vice versa, and (*b*) in starvation of animals have been described.

A study has been made of the McLean-Hastings mass law equation relating serum calcium and serum protein. The unreliability of the constant obtained is pointed out.

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CXCVII. THE PRODUCTION AND UTILIZATION OF LACTIC ACID BY CERTAIN PROPIONIC ACID BACTERIA¹

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LACTIC ACID has long been recognized as a substrate for the propionic acid bacteria. Fitz [1880], the first to present an equation for the propionic acid fermentation, suggested that it might be expressed as



Only recently has this compound been regarded as a possible end product. Foote *et al.* [1930] reported that under certain conditions some cultures of the propionic acid bacteria accumulate this acid. Other investigators [Wood & Werkman, 1937; Fromageot & Tatum, 1933] have reported other conditions which yield similar results. The amount of lactic acid produced varies with pH, nutrients and probably with other factors. The optical properties of the lactic acid produced by these organisms have not been determined. While lactic acid has often been used as a substrate the availability of the optical isomerides of this acid has not been studied.

The present studies were undertaken to determine conditions under which lactic acid would accumulate and to isolate and characterize the lactic acid formed by some of the propionic acid bacteria. The availability of the optical isomerides was also studied.

Cultures and methods

The cultures used in this study were *Propionibacterium pentosaceum*, P₁₁; *P. technicum*, P₁₂; and *P. shermanii*, P₁₉.

The racemizing action of resting cells upon the Ca salt of *d*(-)-lactic acid was determined at pH 6.8 and 5.0 by the method of Christensen *et al.* [1939].

For most of the experiments a medium containing 2% substrate, 0.5% peptone, 0.5% Na acetate, 100 ml. yeast water² and distilled water to make 1 l. was used. 150 ml. of the medium were fermented in 250 ml. Erlenmeyer flasks. In some fermentations sterile CaCO₃ was added immediately before inoculation and in others sterile indicator and alkali were added periodically as required. 1 ml. of a 24–36 hr. culture was used as inoculum. All flasks were incubated aerobically at 30°. Approximately 25 ml. samples for sugar and lactic acid determinations were aseptically removed at the intervals indicated.

Lactic acid was determined by the method of Friedemann & Graesser [1933] upon the ethyl ether extract of an acidified (pH 2) aliquot of the fermented medium. Sugars were determined by the method of Schaffer & Hartmann as modified by Stiles *et al.* [1926].

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² The clear extract from 200 g. pressed yeast autoclaved in 1 l. water.

The basal medium for the study of the availability of the various forms of lactic acid contained 200 ml. yeast water, 0.5 % peptone, 0.004*M* phosphate and distilled water to make 1 l. The substrates *l*(+), *d*(−) and *dl*-lactates and glucose were incorporated in 0.45 % concentrations. The unsupplemented basal medium was also tested. Triplicate tubes of these five media were inoculated with 2 drops of a 5 ml. suspension of washed cells from 10 ml. of a 24 hr. culture which had been grown upon the *dl*-lactate medium. The incidence and rate of growth were determined by following the increase in turbidity as determined with an Evelyn photoelectric photometer.

The specificity of the lactic dehydrogenase was determined by the Thunberg technique. Cells from 24 hr. cultures on the *dl*-lactate medium were washed twice by centrifuging in 0.01*M* pH 7 phosphate buffer and suspended in distilled water in such a concentration that 1 ml. of the cell suspension at pH 6 (the optimum for this dehydrogenase) and 40° would, in the presence of *l*(+)lactate, reduce 1 ml. of 1 : 5000 methylene blue in approximately 5 min. 1 ml. each of the methylene blue solution, 0.04*M* substrate and 0.2*M* pH 6 phosphate-phthalate buffer were placed in the tubes and 1 ml. of the cell suspension was placed in the cap. The contents of the tube and cap were mixed after 3–4 min. equilibration.

Racemization

Racemizing activity could not be demonstrated at either pH 6.8 or 5.0. The absence of this activity suggested that an optically active lactic acid rather than a racemic mixture should be formed by these organisms.

Lactic acid accumulation

Lactic acid accumulation, as indicated by serial analyses of representative fermentations by culture P₁₁ on several media, is shown in Table I. The addition

Table I. *Lactic acid production by P. pentosaceum*

Time and analyses	Glucose					Arabinose	
	CaCO ₃		Neutralized with NaOH		Not neutralized No. 13	CaCO ₃	Neutralized with NaOH
	No. 1*	No. 11	No. 2*	No. 12		No. 3*	No. 14
	Period I						
Days of incubation	4	4	5	4	4	5	4
Sugar fermented, % of total	15.9	42.1	56.1	31.8	15.2	39.5	24.8
Lactic acid, %:							
of utilized sugar	15.9	16.4	16.4	31.2	47.6	2.6	24.8
mg. per 100 ml.	47	33	184	199	145	20	155
	Period II						
Days of incubation	9	7	7	7	8	7	7
Sugar fermented, % of total	77.3	90.2	74.7	50.6	19.6	68.2	46.8
Lactic acid, %:							
of utilized sugar	8.2	1.0	14.9	23.2	40.0	1.5	16.9
mg. per 100 ml.	13	18	222	235	157	20	157
	Period III						
Days of incubation	11	11	10	11	—	10	11
Sugar fermented, % of total	82.2	99.0	80.1	71.2	—	89.3	54.2
Lactic acid, %:							
of utilized sugar	4.7	0.7	11.8	6.1	—	1.0	5.4
mg. per 100 ml.	8	13	189	87	—	18	59

* Medium 2 % sugar, 500 ml. yeast water, distilled water to 1 l.

of CaCO_3 (nos. 1, 11 and 13) to the basal medium resulted in a rapid, almost complete fermentation with a very low lactic acid production. With daily neutralization (nos. 2, 12 and 14) the fermentation was almost as rapid as in the presence of carbonate, while the lactic acid accumulation was markedly increased. The reaction of these fermentations varied from pH 5 to 7. In the unneutralized basal medium high percentages of the utilized sugar appeared as lactic acid but, because of the slow rate of fermentation, the percentage of total sugar converted into lactic acid was less than in the preceding fermentations. Replacement of Na acetate with other buffers, addition of concentrates of propionic growth factors and variation in method of neutralization failed to yield significant increases in yield of lactic acid.

From the data for fermentations 2 and 12, Table I, it will be noted that the concentration of lactic acid is maximal near 7 days. After that time the lactic acid concentration tends to decrease while the percentage of lactic acid, based upon the sugar fermented, steadily decreases throughout the period of incubation. This latter fact, as well as the decrease in total weight of lactic acid, tends to substantiate the theory that lactic acid is an intermediate which is formed during the early stages of the fermentation and later utilized.

••

Isolation of the lactic acid

Four litres of the basal medium were inoculated with 200 ml. of a 36 hr. culture and neutralized daily with NaOH. After 7 days' incubation at 30° the medium was concentrated to 400 ml., acidified to pH 2 and extracted with ethyl ether for 48 hr. Propionic and acetic acids were removed from this extract by steam distillation and the neutralized (pH 6.5) residue was treated with Ag_2O to remove succinic acid. The remaining solution was acidified and the lactic acid extracted with ethyl ether. The Zn salt of the lactic acid was prepared and purified by recrystallization from 50% alcohol.

The data, Table II, show that both species form *l*(+)lactic acid and that the optical configuration is independent of the sugar fermented.

Table II. *Properties of Zn salts of lactic acid*

Organism	Sugar	Water of crystallization %	Zn* content %	Specific rotation†
P ₁₁	Glucose	12.85	33.55	-8.26
P ₁₁	Arabinose	12.92	33.40	-8.26
P ₁₂	Glucose	12.87	33.45	-8.23
Zn salt of <i>l</i> (+)lactate		12.89	33.40	-8.25
Zn salt of <i>dl</i> -lactate		18.18	33.40	0.00

* Value for anhydrous salt.

† Determined in 4% concentration.

Utilization of the optical isomerides of lactic acid

In view of the production of only one form, the *l*(+)acid, it seemed of interest to determine the availability of each form of lactic acid to these organisms. The growth of *P. pentosaceum* upon glucose, the isomeric lactates, and the basal yeast-water, peptone, phosphate medium is indicated in Fig. 1. Photometer readings have been converted into millions of cells per ml. to facilitate comparison.

Development was more rapid on the *l*(+)lactate than on the *d*(-)lactate while the racemic lactate supported faster growth than did either of the optically active forms. The differences in cell count were greatest at 45 hr. A greater rate of growth and approximately double total growth proved glucose to be a better medium than any form of lactate in the same concentration.

The growth of *P. shermanii* and *P. technicum* upon these media was identical with that of *P. pentosaceum*. The differences between the rates of growth upon the *l*(+) and *d*(-)lactates were approximately the same for each culture. Although these differences were small, they are considered significant because in each of the three cultures they were much greater than the differences between

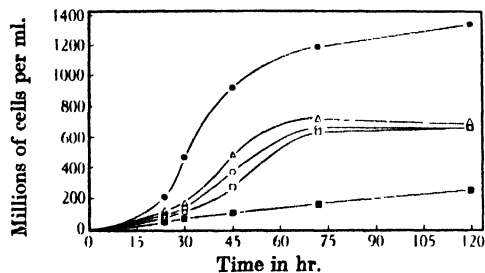


Fig. 1. The growth of *P. pentosaceum* upon glucose and the optical isomerides of lactic acid.

● Glucose. Δ *dl*-Lactate. ○ *l*(+)-Lactate. □ *d*(-)-Lactate. ■ Basal medium.

triplicate tubes. The ultimate level of growth seems to be independent of the optical form of the acid and exhibits a remarkable constancy of magnitude for not only the three acid forms, but also for the three cultures. The average of the final cell counts upon the three lactate media were *P. pentosaceum*, 698; *P. technicum*, 717; and *P. shermanii*, 709 million cells per ml.

Because of the differences in the growth rates upon the optical isomerides of lactic acid the activity of the lactic dehydrogenases of these species of the propionic acid bacteria was investigated. These data are shown in Table III. The

Table III. Activity of the lactic dehydrogenases of propionic acid bacteria

Substrate	Culture	Cell activity*		
		P ₁₁	P ₁₂	P ₁₁
Endogenous		0.000	0.000	0.000
<i>l</i> (+)Lactate		0.505	0.154	0.203
<i>dl</i> -Lactate		0.295	0.100	0.193
<i>d</i> (-)Lactate		0.068	0.072	0.091

* Reciprocal of decoloration time in min.

results are expressed in suspension activities, i.e. the reciprocal of the observed decoloration time expressed in minutes. No endogenous respiration was detectable in these cell preparations within 18–24 hr. These data indicate the presence, in each of the organisms studied, of specific *l*(+) and *d*(-)lactic dehydrogenases. As suggested by the growth response, the activity of the *l*(+)lactic dehydrogenase of each organism is significantly greater than that of the *d*(-)enzyme. The activity upon the racemic substrate, however, is intermediate.

SUMMARY

1. The influence of certain cultural conditions upon lactic acid accumulation was studied.

2. *P. pentosaceum* produced only *l*(+)lactic acid from glucose and arabinose and *P. technicum* only *l*(+)lactic acid from glucose.

3. *P. technicum*, *P. pentosaceum* and *P. shermanii* failed to racemize optically active lactic acid.

4. The growth rates upon the optical isomerides of lactic acid varied in the order $d(-) < l(+) < dl$. The ultimate level of growth was the same in each case.
5. The presence of both $l(+)$ and $d(-)$ lactic dehydrogenases in these organisms was demonstrated. The $l(+)$ enzyme possessed the greater activity.

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CXCVIII. THE ROLE OF SORBITOL IN THE CARBON-METABOLISM OF THE KELSEY PLUM

I. CHANGES IN CHEMICAL COMPOSITION DURING GROWTH AND STORAGE

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MUCH of the C lost as CO_2 by respiring fruit may usually be accounted for in terms of loss of sugar and acid. But this does not appear to be true for mature Kelsey plums which, though they contain no starch, were found on storage at 13 or 25° to show no loss of sugar at all, and under certain conditions of storage even showed an increase in sugar content.

On investigation it was found that Kelsey plums contain a considerable amount of sorbitol, in some instances as much as 4.5 % of the fresh weight in the mature fruit. Quite a number of the fruits of the Rosaceae are now known to contain sorbitol [Reif, 1934; Strain, 1937], but little is known of the role of hexitols in the metabolism of higher plants. That sorbitol is a probable source of sugar for respiration has been indicated by some investigators [Nuccoroni & Bartoli, 1932; Martin, 1937]. In the Kelsey plum there appears to be also a definite relationship between the initial sorbitol content and the fruit's susceptibility to low temperature injury in store [Donen, 1939]. The part played by sorbitol thus appears to be not only of theoretical but also of some commercial importance.

The investigation described in this paper deals with the accumulation of sorbitol in the Kelsey plum during growth, and traces the course of change in the respirable material of the fruit when stored at 13 and 25° and the extent to which this is modified by a previous exposure of the fruit to 1°. The quantitative relation between loss of C as CO_2 and C as carbohydrate and acid will be discussed in a later communication.

EXPERIMENTAL PROCEDURE

Plan of experiments. Changes during growth were estimated by collecting weekly samples of plums over a period of 77 days before the commercial gathering of the fruit in the 1938-9 season. At each weekly picking three extra samples were taken for storage at 25°, and these were analysed at fortnightly intervals. The general effect of maturity on loss of sorbitol and sugar in store was thus determined. These data were used to corroborate more detailed studies on chemical changes in plums stored at 13° carried out in 1937-8 on three series of samples (S1, S2 and S3). These series were chosen to represent fruit of contrasting sorbitol and sugar contents, the picking being made at 90, 120 and 154 days after petal-fall, when the mean weights per fruit for the three series were 22.4, 50.2 and 80.0 g. respectively. The last pick (S3) represented fully mature Kelsey plums.

In addition two further series of samples (S4 and S5) were picked in 1937-8 for storage at 1°. S4 was similar to S3, but S5 consisted of plums from a different

group of trees, on which, in contrast to the first group, the crop had not been thinned and the plums were expected to be of very low sorbitol content.

Method of sampling. Each sample was picked separately by a method of random selection. Plums for S1, S2, S3 and S4 were picked off the same 16 trees on a farm in Somerset West, Cape. S5 was taken from a different group of trees in the same orchard. The samples for estimation of changes during growth and storage at 25° were taken from a group of 20 trees on a farm in Banhoek. A sample usually consisted of 48 plums, but for mature fruit it was reduced to 32. Each tree contributed the same number of plums to every sample.

The trees on both farms were well grown, about 11 years old, well irrigated and fertilized and carrying a moderately heavy crop.

Preparation of sample. Immediately after picking the samples were weighed and taken to the laboratory. The initial samples of each series were analysed immediately, the others stored in constant temperature chambers maintained at high R.H. (90–95 %). Loss of weight was estimated by weighing the fruit again just before analysis.

For analysis the plums were quartered, stones removed, and two opposite quarters of each plum put through a mincing machine. The pulp was thoroughly mixed and 250 g. taken for extraction with 75 % alcohol [Donen, 1937]. After extraction the alcohol was evaporated *in vacuo* at 30° and the aqueous solution made up to 250 ml. and filtered through kieselguhr. Tests showed that filtration through kieselguhr in no way affected the results of analysis.

Methods of analysis

Alcohol-insoluble residue (A.I.R.) was obtained by collecting the residue from alcohol extraction and drying at 80° for 100 hr.

Dry weight was estimated on a portion of pulp dried at 50° for 84 hr. in a well-ventilated oven.

Soluble solids (i.e. solids which are soluble both in 75 % alcohol and in water). 5 ml. of extract as obtained above were dried in a shallow, flat-bottomed dish at 50° for 84 hr. Excellent agreement was obtained between values of dry weight and the sum of soluble solids plus A.I.R.

Acidity. 5 ml. of extract were diluted to 250 ml. with distilled water and titrated with *N*/10 NaOH, with phenolphthalein as indicator. Results were expressed in terms of malic acid.

Residual soluble solids (R.S.S.) were obtained by calculation:

$$\text{R.S.S.} = \text{soluble solids} - \text{sugar} - \text{acid} - \text{sorbitol.}$$

Sugar analysis. Harding and Downs modified copper reagent was used, and the procedure adopted for the determination of reducing sugar, sucrose and fructose was exactly as laid down by van der Plank [1936]. The original extracts were not clarified with Pb acetate or charcoal; baker's yeast was employed instead, and the reducing value of the extracts after fermentation was used as a correction for interference by substances other than sugar. This correction was small, usually 0.2 %, as compared with the total sugar value of 5–10 % of fresh weight. Results obtained in this manner agreed well with those obtained on extracts previously clarified with dibasic Pb acetate.

Sucrose was hydrolysed by adding 2.5 ml. of 5*N* H₂SO₄ to 25 ml. of approximately 0.2 % sugar solution and then heating at 70° for 12 min. On cooling the acid was neutralized with a previously estimated quantity of 10*N* NaOH. Tests were carried out on 13 plum extracts to compare the results of inversion of sucrose by the above method and by using invertase. Close agreement was obtained, although acid hydrolysis usually gave slightly higher results. The mean difference

in 13 tests was $+1.1\%$ and the highest difference 1.8% . As a further check on the above method, estimations were made of increase in fructose after hydrolysis with acid and after inversion with invertase. Identical results were obtained. Calculation of the ratio of increase of fructose to increase of glucose on hydrolysis gave a value of 1.039 ± 0.021 (mean of 10 determinations). As this calculation carried the error of four separate sugar determinations, the result is most satisfactory and leaves no doubt that only sucrose is hydrolysed by treatment with H_2SO_4 .

Isolation and identification of sorbitol. 100 ml. of plum extract were fermented with 2.5 g. of yeast at 37° for 72 hr. The yeast was removed by centrifuging and then by filtration through kieselguhr. A portion equivalent to 5 ml. of original extract was evaporated in a 100 ml. beaker to a syrup, cooled and treated with 0.5 ml. of benzaldehyde and 1 ml. of 50% (by vol.) H_2SO_4 . On stirring for a few minutes a buff-coloured syrupy mass was obtained which, on storing at 1° , set to a cake within a few hours. The procedure followed thereafter was the same as described by Martin [1937] for determination of sorbitol. All his precautions were observed. A whitish granular powder was obtained, M.P. $172\text{--}174^\circ$. This corresponds to the M.P. of dibenzalsorbitol as commonly reported. The benzal derivative was hydrolysed with HCl, and the acid and benzaldehyde removed by steam distillation. The residue was acetylated and recrystallized as described by Tutin [1925]. A small crop of well-formed crystals was obtained which melted at 97° and was found to be identical with hexa-acetylsorbitol.

A portion of the fermented extract was evaporated to dryness and treated with pyridine as described by Strain [1934]. A large crop of white crystals was obtained. This was recrystallized several times from pyridine, then dried *in vacuo* over conc. H_2SO_4 for several weeks and finally exposed to the air of the laboratory for 7 days. By then the crystals had lost all their pyridine and a white granular cake remained which melted at $91\text{--}92^\circ$ and was found to be identical with anhydrous sorbitol [Strain, 1934].

Estimation of sorbitol. That sorbitol may be obtained in best yields as the dibenzal derivative is well known. Martin [1937] claims that by adopting certain precautions and slightly modifying Werder's original procedure [1932] for estimation of sorbitol, nearly theoretical yields of dibenzalsorbitol may be obtained. Furthermore he claims that sugar does not interfere with the determination.

Martin's claims were substantiated by tests on pure sorbitol and on plum extracts. Good replication was obtained. Thus: taken 0.1906 g. sorbitol, found (by the dibenzal method) 0.1937 ± 0.0054 g. (mean of six determinations). This represents a yield of $101.6 \pm 2.8\%$. Since some of the available plum material had been previously dried at 50° , tests were also made to find out whether drying of plum pulp at that temperature affected the subsequent yield of sorbitol. 5 g. of the dried material were extracted five times with 75% ethyl alcohol at 60° and the alcohol removed by evaporation *in vacuo*. Portions of the aqueous solution were used for sorbitol determinations. The results (Table I) indicate that drying

Table I

Sample	Fresh pulp. Sugar removed by fermentation	Fresh pulp. Sugar not removed	Dried material. Sugar not removed
1	0.97	—	0.89
2	1.99	—	1.91
3	2.56	—	2.51
4	2.04	2.06	1.97
5	2.71	—	2.68
6	1.50	1.52	—

of the pulp at 50° and the presence of sugar in the extract did not materially affect the yield of sorbitol.

In this study sorbitol was determined as the dibenzal derivative on alcoholic extracts of the dried material or, when available, on that of fresh pulp. Martin's procedure was adhered to, but the precipitates were dried at 80° instead of 100°.

Presentation of results and error of estimation. All results are expressed as percentage of original fresh weight (o.f.w.) and are presented in a series of graphs. In Figs. 4-7 curves of best fit were calculated to the experimental points. Most of these curves are straight lines, but some, e.g. the sorbitol curve in Fig. 6, are exponential in form. Eight samples for initial analysis were taken with each series of storage samples and the results used for calculation of standard error and significant differences. Standard deviation was calculated as $\sigma = \sqrt{\frac{ev^2}{n-1}}$ where v = deviation from mean and n = number of samples. Significant differences were taken as 2.31σ and are indicated by lines along the appropriate curves in the figures given in the text.

DISCUSSION OF RESULTS

Sorbitol accumulation during growth

The chief point of interest in the accumulation of sugar-alcohol in Kelsey plums during growth is its relationship to sugar intake. Even very young fruits contain as much as 1% of sorbitol when total concentration of sugar is only 4% and when sucrose is almost entirely absent (Fig. 1). Rapid accumulation of the sugar-alcohol begins only during the period of cell enlargement just after stone growth is completed. The beginning of this period usually coincides with the minimum concentration of dry weight of the fruit [Donen, 1936].

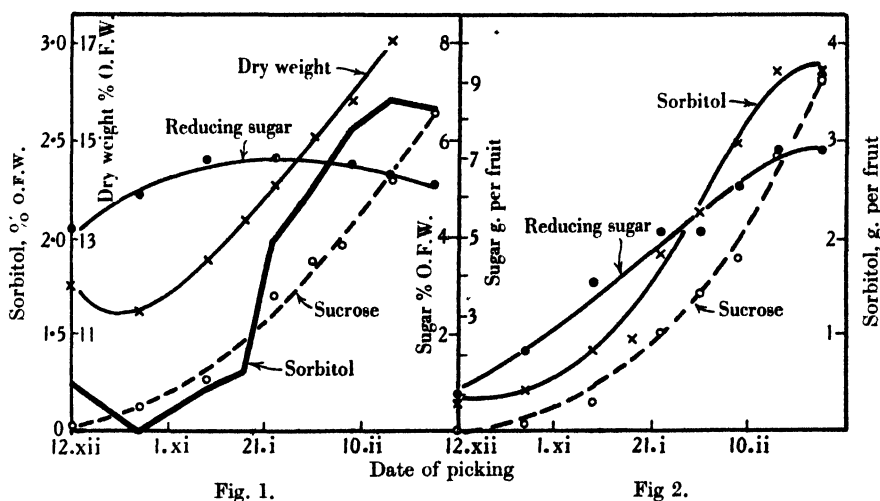


Fig. 1.

Fig. 2.

Fig. 1. Change in concentration of dry weight, sugars and sorbitol in the Kelsey plum during growth.

Fig. 2. Accumulation of sugar and sorbitol in the Kelsey plum during growth.

A possible interrelationship between sorbitol and the hexoses, but not sucrose, is strongly suggested by the concentration curves shown in Fig. 1. The shapes of the curves for reducing sugar and sucrose are typical for these sugars and have been confirmed over a number of seasons and for fruit from different

orchards [Donen, 1936]. It is significant, therefore, that any notable increase in sorbitol concentration should occur only when the concentration of reducing sugars has reached a constant level. This suggests that sorbitol is stored instead of hexoses when the latter have reached their maximum concentration. If hexoses are then accumulated at a greater rate than the rate of growth of the plum, i.e. in excess of the amounts needed to maintain the hexose concentration, sorbitol will be formed. Towards the end of the growth period, when hexose accumulation (g./fruit, Fig. 2) declines, sorbitol formation also stops. On the other hand sucrose continues to accumulate so long as the plum remains on the tree [Donen, 1936].

That the fall in intake of sorbitol towards the end of the growth period is genuine and not due to sampling error was confirmed by analysis of a series of samples of plums from different orchards picked just at the first appearance of colour in the fruit and again about a fortnight later when the plums were showing considerable yellowing and reddening—the usual signs of advanced ripening. The results (Table II) indicate that sorbitol concentration shows little change towards the end of the growth cycle of the plum.

Table II

Sample	1	2	3	4	5	6	7
Sorbitol concentration as % O.F.W.:									
Fruit with 1st tinge of red			2.23	1.50	1.61	3.15	2.10	1.87	2.92
Ripe fruit			2.11	1.45	1.56	3.24	2.30	1.98	2.60

Sources of respirable material

The mature Kelsey plum contains, on the average, about 16.65 g. of dry weight material per 100 g. fresh weight.¹ Of this 1.5 g. represent the alcohol-insoluble residue which contains no starch and consists mostly of hemicelluloses and pectic materials. Widdowson [1932] found no substantial evidence that the hemicelluloses present in the apple are a source of reserve carbohydrate in that fruit. It is probable that this is also true for the plum, for the observed loss of A.I.R. in the plum on storage is very small and only just larger than the calculated significant difference (Figs. 4-6).

The 15.15 g. of alcohol-soluble solids consist of 10.48 g. of sugar, 2.82 g. of sorbitol and 0.835 g. of acid (expressed as malic). This leaves 1.01 g. of residual soluble solids which should be almost completely accounted for by soluble nitrogenous compounds (equivalent to 0.07 g. N, i.e. very approximately 0.35 g. organic material) and soluble mineral salts (equivalent to 0.4 g. of ash). The changes in R.S.S. fraction in the Kelsey plum on storage are very small (Figs. 4-6) and not significant. The general tendency of the R.S.S. curves to show an increase and for those of A.I.R. to show a decrease during storage might be partly due to the progressive hydrolysis of proteins [Donen, 1936] and also to the increase in the soluble pectin fraction in stored plums. Under the conditions of alcoholic extraction employed, it became increasingly difficult to precipitate all of the soluble pectin.

It thus appears that in the plum the likely source of respirable material during storage is confined to sugars, acid and sorbitol. This is borne out by results given in Table III which show that total loss in dry weight is very nearly equal to the sum of losses in acid, sugar and sorbitol.

¹ The figures quoted are average results obtained by taking mean values of 44 analyses of commercially ripe fruit from different orchards.

Table III. *Losses of respirable material from stored plums and the probable errors of the various estimates*

All results in g. of original fresh wt.

Popula- tion	Days	Loss of dry wt.	Loss of sorbitol So	Loss of acid A	Loss of sugar S_T	Total loss of So + A + S_T	Loss of So + A + S_T as % loss of dry wt.
S1	64	1.92 ± 0.10	0.39 ± 0.03	0.70 ± 0.08	0.81 ± 0.09	1.90 ± 0.12	99 ± 9
S2	102	4.16 ± 0.15	0.89 ± 0.03	1.22 ± 0.03	1.86 ± 0.08	3.97 ± 0.09	95.5 ± 4
S3	55	2.80 ± 0.14	2.14 ± 0.11	0.46 ± 0.03	0.00 ± 0.15	2.60 ± 0.17	93 ± 8
S4 (1-20°)	49	2.09 ± 0.14	1.53 ± 0.12	0.35 ± 0.03	-0.09 ± 0.15	1.79 ± 0.20	86 ± 12
S4 (1-7.5°)	68	1.68 ± 0.14	1.46 ± 0.12	0.52 ± 0.03	-0.33 ± 0.15	1.65 ± 0.20	98 ± 15
S5 (1-20°)	49	3.09 ± 0.13	0.89 ± 0.04	0.53 ± 0.02	1.48 ± 0.10	2.90 ± 0.11	94 ± 6
S5 (1-20°)	68	2.35 ± 0.13	1.09 ± 0.04	0.51 ± 0.02	0.74 ± 0.10	2.33 ± 0.11	99 ± 8

Loss of respirable material

The loss of sorbitol from plums stored at 25 or 13° is very rapid and the rate of initial loss increases markedly with maturity (Fig. 3). On prolonged storage the sorbitol content reaches a very low and almost constant value, and usually at this point there is a notable increase in the rate of loss of total sugar (Figs. 3-7). This interrelationship between sugar and sorbitol is shown in all series of samples. The young fruits of S1, which contained very little sugar-alcohol, show an immediate loss of sugar; plums with a relatively low sorbitol content (S2) first lose practically all of their sorbitol before any marked fall in sugar content is observed;¹ whilst plums with a high concentration of sorbitol (S3) show a rapid loss of sugar-alcohol but hardly any loss of sugar. This contrast between fruits of high and low sorbitol contents is again shown by the S4 and S5 plums (Fig. 7).

The rate of acid loss shows no marked relation to maturity and does not in any way reflect the rate of loss of either sugar or sorbitol. The changes in acid in all series follow, however, the dry weight curves which are quite a good measure of change in total respirable material (Table III).

The total sugar values obtained in samples 7, 8 and 9 (Fig. 3) and those for S3 (Fig. 6) strongly suggest that in mature plums stored at 13 and 25° sugar content remains constant if sorbitol concentration is high. A straight line has therefore been fitted to the total sugar curve in S3. The mean value for total sugar in all stored samples of this series is 10.15 ± 0.22 and the value calculated for eight initial samples is 10.10 ± 0.15 . No significant loss of sugar has therefore taken place.

Effect of storage at low temperature

When Kelsey plums are first stored at low temperature (0 or 1°) for 21-25 days and then raised to higher temperatures (above 7.5°) they show an increase of as

¹ The greatly increased rate of sugar and acid loss which occurs in S2 (Fig. 5) on, say, day 40 is not entirely due to depletion of sorbitol. The onset of the respiratory climacteric occurred at that stage, and the marked loss of acid and sugar as well as of dry weight is a reflexion of the increase in rate of respiration. Sorbitol exhaustion does not normally coincide with onset of the climacteric.

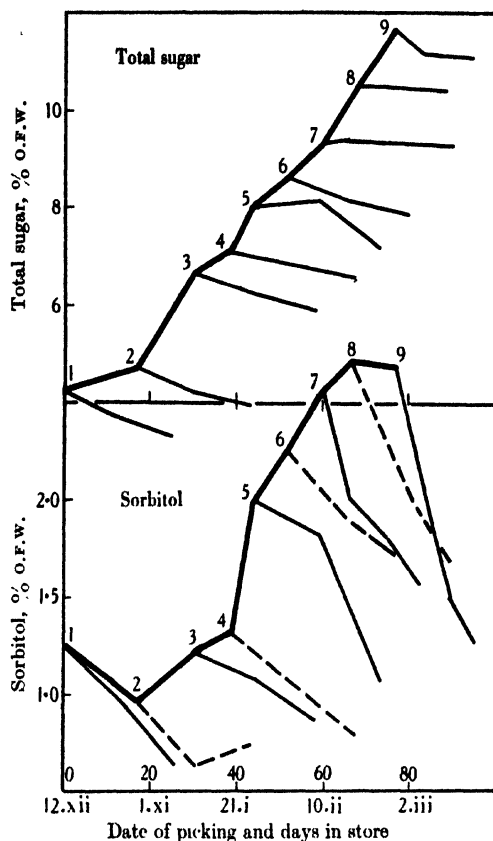


Fig. 3.

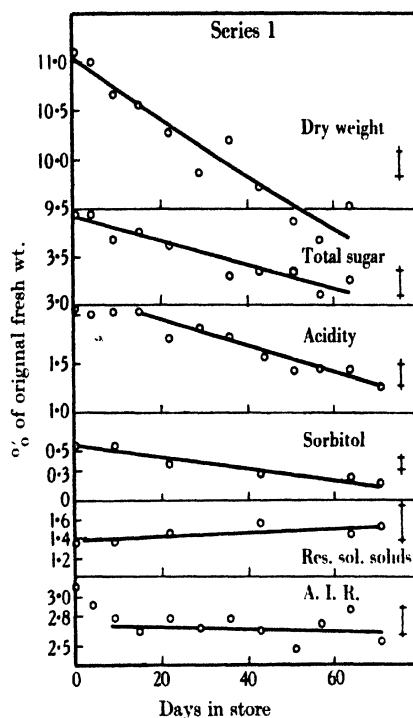


Fig. 4.

Fig. 3. Effect of maturity on loss of total sugar and sorbitol in Kelsey plums stored at 25°. Heavy lines refer to initial analyses on picking, thin lines to analysis in store. Numbers along the heavy lines indicate serial number of picking.

Fig. 4. Chemical changes in Kelsey plums stored at 13°. S1, vertical lines alongside curves indicate significant differences.

much as 10–15% of total sugar. Some typical results are given in Table IV; the observed increase in sugar is always accompanied by a slightly greater decrease in sorbitol.

Table IV. *Changes in total sugar and sorbitol after exposure to low temperature*

Sample	Initial analysis		25 days at 0°		25 days at 0° and 10 days at 25°		25 days at 0° and 20 days at 25°	
	Sugar	Sorbitol	Sugar	Sorbitol	Sugar	Sorbitol	Sugar	Sorbitol
1	10.68	3.42	10.76	—	11.28	2.20	11.04	1.74
2	10.43	3.79	—	—	10.80	—	11.67	2.11
3	11.28	3.44	11.58	2.63	12.35	2.47	11.96	2.14
4	10.63	3.52	—	—	11.37	2.55	11.81	2.35
5	9.93	3.27	—	—	11.15	—	10.14	2.07
6	10.88	3.20	—	—	12.20	1.89	11.72	2.08
7	10.89	2.67	—	—	11.82	1.43	10.18	0.99

The relationship between sorbitol content and sugar increase was studied by storing two series of plums (S4, high in sorbitol; and S5, low in sorbitol) at first for 25 days at 1° and then one set of each series at 20° and another set at 7·5° (Fig. 7). The increase in sugar begins in the plum during the period of exposure to low temperature and the extent of the increase depends upon the initial sorbitol content and the rate of its depletion. When the plums are transferred from low to

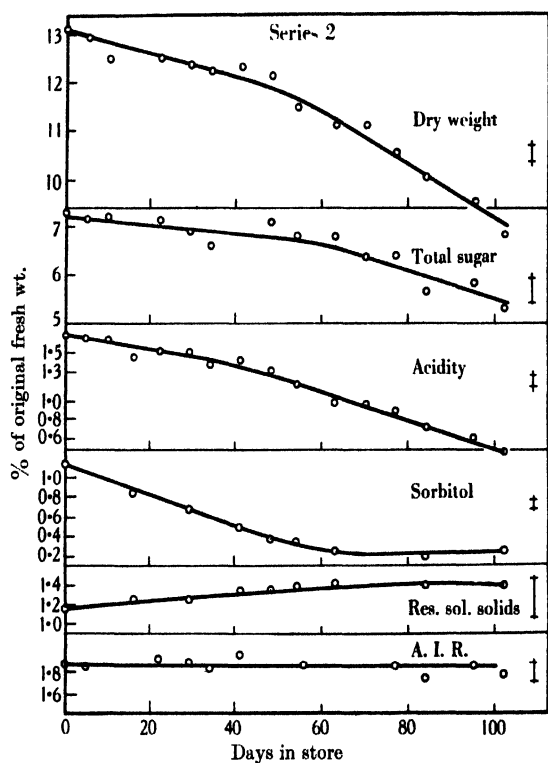


Fig. 5.

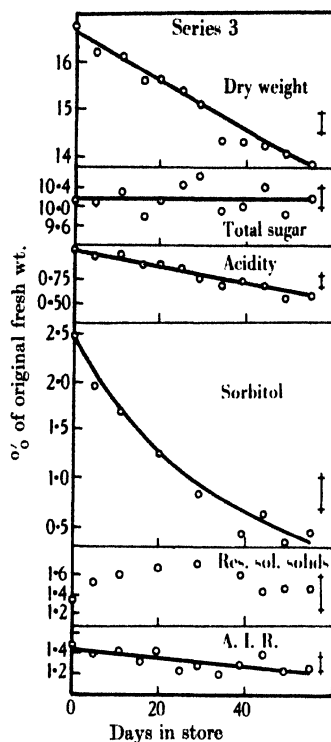


Fig. 6.

Fig. 5. Chemical changes in Kelsey plums stored at 13°. S2, vertical lines alongside curves indicate significant differences.

Fig. 6. Chemical changes in Kelsey plums stored at 13°. S3, vertical lines alongside curves indicate significant differences.

higher temperatures the rate of sorbitol loss is greatly increased. The increase in sugar continues, however, until the concentration of sorbitol has become very low. After that, rapid loss of sugar may occur, as in S5, where the sorbitol reached a very low and almost constant level. Plums of S5, containing only 1·3 % sorbitol, showed much less increase in sugar than the S4 fruit which initially had 2·5 % of sugar alcohol.

Martin [1937] reported that Bosc pears when ripened at 67° F. lost sorbitol and showed an increase of sugar. He did not emphasize that his pears were originally stored at 30–31° F. for about 9 weeks, and apparently assumed that sorbitol gave rise directly to sugars. If that is also true for the Kelsey plum the observed constant value for total sugar in the S3 would then be merely a reflexion of the balance between sugar lost in respiration and sugar replaced by sorbitol.

In the next communication it will be shown, however, that total loss of C as sugar, acid and sorbitol (and occasionally as sorbitol alone) considerably exceeds the observed loss of C as CO_2 during the first 30 or 40 days of storage of plums at 25 or 13°. Under these conditions accumulation of sugar should have been observed. Increase in sugar was observed, however, only in those plums which had been previously kept for some time at low temperature, whilst good agreement between loss of C as CO_2 and C lost as sorbitol + sugar + acid was obtained only if the plums were kept in store for some considerable time (60–100 days).

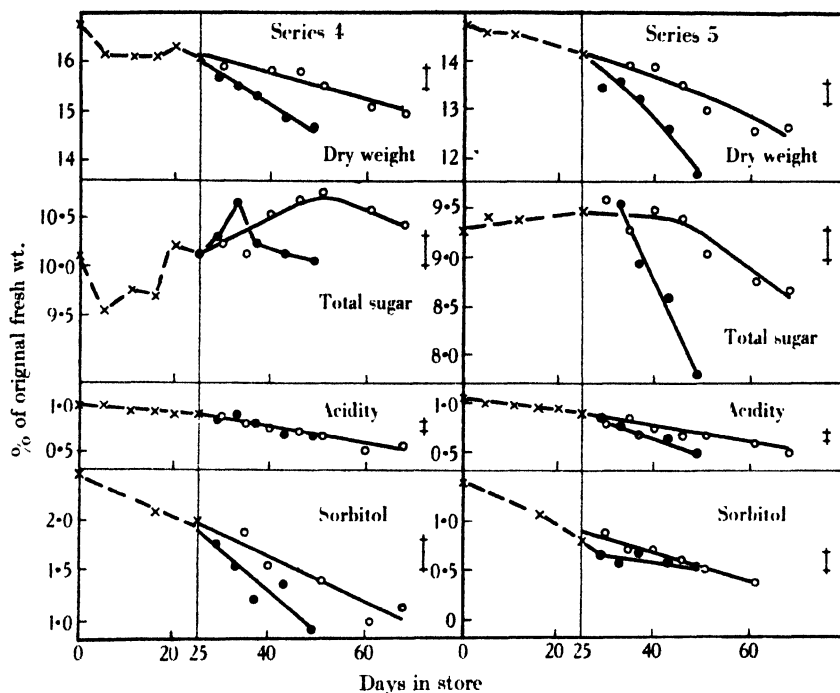


Fig. 7. Chemical changes in Kelsey plums stored for 25 days at 1° and then transferred to 20 and 7.5°: —x—x—x— 1°, —●—●—●— 20°. Vertical lines alongside the curves indicate significant differences.

This suggests that possibly sorbitol first breaks down to intermediate compounds, which under certain conditions (e.g. exposure to low temperature) are rapidly converted into sugars.

SUMMARY

1. Sorbitol has been identified as a constituent of the Kelsey plum.
2. Experiments are described tracing the accumulation of sorbitol during growth and the changes that take place in the respirable material of the plums on storage at 13 and 25°.
3. The mature Kelsey plum contains, on the average, 2.8% of sorbitol, most of which is accumulated during the later part of its growth cycle. It is suggested that during growth of the plum sorbitol is stored in place of hexoses when the latter have reached a maximum concentration.
4. Loss of respirable material from stored plums can be almost completely accounted for in terms of sorbitol, sugar and acid. Sorbitol is lost rapidly in

store, but the loss of sugar is dependent upon the initial sorbitol concentration. At 13 and 25° mature plums of a high sorbitol content show no significant loss of sugar. Plums of low sorbitol content show marked sugar loss only when most of the sorbitol has disappeared.

5. When plums are stored at 1° for 25 days and then transferred to 7·5 or 20° they show a 10–15 % increase in total sugar. The extent of this sugar increase depends upon the initial sorbitol content and on its rate of exhaustion in store.

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CXCIX. CRITICISM OF SHUTE'S SERUM TEST FOR VITAMIN E DEFICIENCY

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IN 1935 Shute described a simple test which, he claimed, showed that the blood of aborting women contains a factor which inhibits proteolytic digestion. He was inclined to relate this finding to the actual abortion by postulating that the factor in question was responsible for increasing the resistance of the uterine walls to penetration by the placental villi [Shute, 1935, 1]. Investigating the matter further [Shute, 1935, 2] he found that the substance responsible for this effect could be removed from sera by chloroform extraction. From his observation that a normal rate of "proteolysis" was shown by sera withdrawn a few days after abortion, he was led to think that the placenta might be the source of the factor in question. Suspecting a fat-soluble substance in placenta he made experiments with certain sex hormones and found that the addition of oestrogens to normal sera produced an increased antiproteolytic action similar to that shown by the sera from cases of abortion.

Another important link in the chain of evidence which led him to put forward a theory regarding the physiological role of vitamin E was his observation that the sera of rats which were known to be in a condition of marked vitamin E deprivation showed similar behaviour to those of aborting women or normal sera to which oestrogens had been added [Shute, 1936]. Moreover, the response to the test became similar to that of normal sera after the vitamin E-deficiency condition had been successfully treated with wheat germ oil. On the basis of this experimental work he has put forward the view that "vitamin E and oestrin, or a substance much like it, exist in a sort of equilibrium during pregnancy. If there is too much of the oestrin-like substance the pregnancy is interrupted."

We are not here concerned with physiological criticisms of his theory; these are the subject of another communication [Drummond *et al.* 1939]. We are now reporting our inability to obtain results similar to his when comparative experiments were made with sera of normal rats, of others in a condition of vitamin E deficiency and of a group that had been fed on a diet containing a large excess of vitamin E.

In the first place, there are a number of points which appear unsatisfactory in his description of the test [Shute, 1935, 1:1938]. Briefly, the test consists in placing 0.5 ml. of serum diluted with 3.5 ml. of a borax-sodium dihydrogen phosphate buffer of pH 9 in each of two tubes, one of which is heated to $55 \pm 5^\circ$ for 30 min. At the end of this time the tubes are cooled, a known amount of trypsin solution is added in equal amounts to each and the contents are incubated at a temperature between 37 and 42°. At intervals of 10 min. 0.5 ml. is withdrawn from each tube and, after dilution with 5 ml. of distilled water, is titrated with *N*/70 or *N*/100 NaOH to pH 9, using phenolphthalein as indicator. The titrations are regarded as a measure of increase in acidity due to "proteolysis", but Shute admits that

under the conditions of his test there is no increase in the formaldehyde titration. It is difficult to reconcile the view that a change in the reaction of the contents of the tube is due to "proteolysis", presumably due to the trypsin, with his statement that the "digestion" takes place over a range of temperature from 20 to 90°. His object in heating one tube to 55° for $\frac{1}{2}$ hr. is not, as one would expect, to have a control in which the antitryptic factor is inactivated, but, rather surprisingly, because he sometimes finds that the preliminary heat treatment gives a more definite result in the final test. He does not offer any explanation of this curious observation. Equally disturbing is his view that the digestion of the serum-buffer mixtures by trypsin under these conditions takes place according to an "all-or-none" principle, whilst the fact that he sometimes finds a "reversal" of his titration values in the later stages of the test is another observation which is difficult to explain. Of this "reversal" he himself remarks that it "seems to have eluded satisfactory explanation".

EXPERIMENTAL

Our first attempts to employ Shute's test as a means of differentiating between the sera of vitamin E-deficient and normal rats failed completely, although we followed as carefully as possible the details of the method as set out in his papers.

Fortunately, at this stage of the investigation we had the opportunity of availing ourselves of Dr Shute's personal advice and of seeing him carry out a demonstration of his method. For this willing co-operation we are greatly

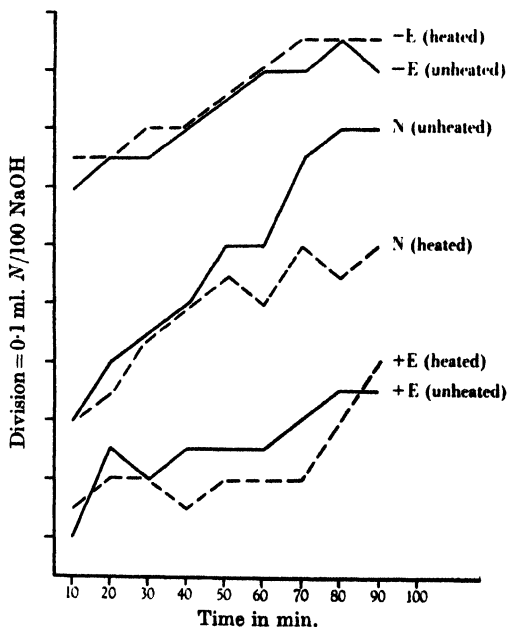


Fig. 1.

indebted. In the course of discussion he agreed that the end-point of the titrations, which in his publications are stated to be "easily read", would be improved by the choice of a more appropriate indicator than phenolphthalein, it being borne in mind that a strong buffer mixture is being titrated from pH 8 to 9.

A much more suitable indicator was found in one of the methyl-red-methylene blue-phenolphthalein achromatic indicators described by Smith [1930], the one chosen showing a neutral grey tint at pH 9 and being sensitive to changes of

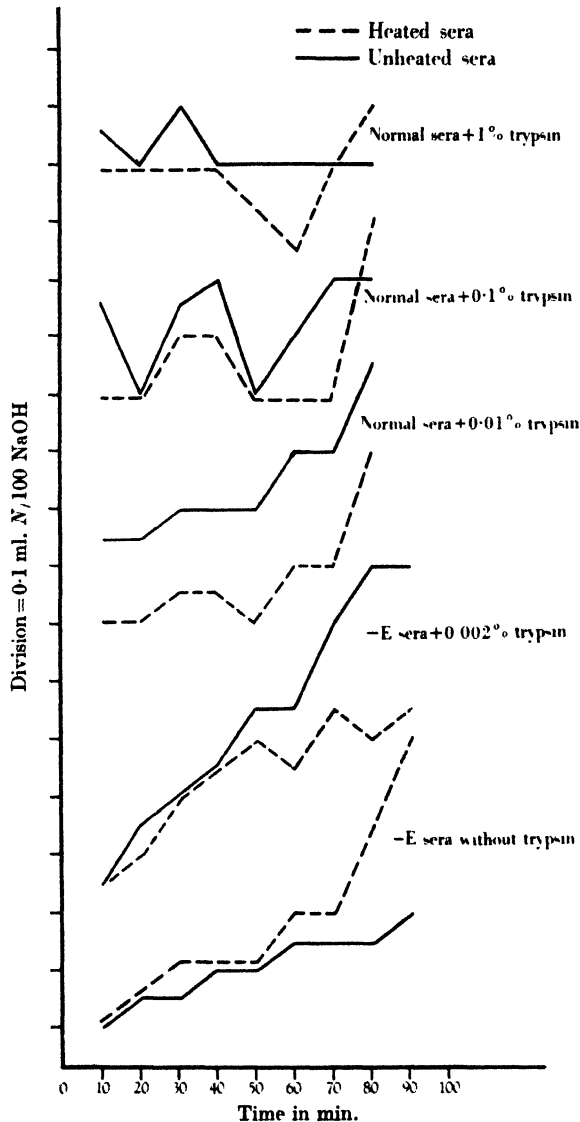


Fig. 2.

0.05 pH . With this indicator we have carried out a considerable number of experiments, following in all other respects the procedure laid down by Shute. The sera examined have been taken from rats (a) fed on an adequate, normal ration (N); (b) fed for from 9 to 12 months on a vitamin E-deficient diet (-E); and (c) fed for 6-9 months on a normal ration supplemented with 12% of wheat germ oil (+E).

The results fail to show any consistent differences. Typical titration curves are shown in Fig. 1.

These experiments were all carried out with a trypsin solution prepared according to Shute's method, namely 1 ml. of a 1% "Difco" trypsin in 75% glycerol diluted to 500 ml. with water. It was thought desirable to repeat the experiment with a stronger solution of the enzyme. Accordingly, a similar series of tests were made employing the stock 1% trypsin and dilutions of 1:10 and 1:100. The titration curves were exactly similar to those obtained when the greater dilution of 1:500 had been used. Much the same types of curves were obtained when the trypsin was omitted (Fig. 2).

This made us suspect that the test had little significance from the standpoint of vitamin E, but it seemed desirable, nevertheless, to repeat the experiment in which Shute found that oestrogens added to normal sera produced an inhibition of "proteolysis". Following closely his procedure we added 8-80 R.U. of "Theelin" (Parke Davis) per ml. to normal rat sera. The titration curves were not materially different from those of the controls (Fig. 3).

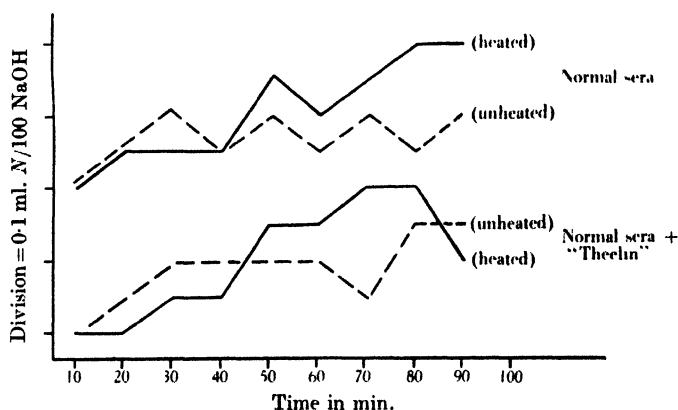


Fig. 3.

The general impression we formed from these experiments was that any small differences encountered were more probably due to absorption of CO_2 than to interaction of the trypsin and the serum. In order to clear up this point we carried out a test on a larger scale using four times the quantities employed in previous tests and taking special precautions against contact with air. We found no significant change in the reaction of the mixture during a period of 2 hr. 20 min., either when normal serum or that from a vitamin E-deficient animal was employed.

Parallel with this series of tests we carried out a number of experiments to ascertain whether there was a real increase in antitryptic action of the serum in vitamin E deficiency. For this purpose we employed a viscosimetric method. Mixtures were prepared with 2.4 ml. of 3.5% solution of gelatin in Clark and Lub's buffer pH 8, 0.1 ml. of serum and 0.5 ml. of *liquor trypticus* (B.D.H.). These were placed in an Ostwald viscosimeter in a thermostat at 40° and records were taken of the viscosity of the fluid over a period of 2 hr. In each experiment a comparison was made between a sample of serum which had been heated to 55° for $\frac{1}{2}$ hr. and an unheated specimen. The curves seen in Fig. 4 show that there is no significant difference between the rates of digestion of gelatin by trypsin in

the presence of normal rat serum and of serum taken from rats either deprived of vitamin E or fed liberally on wheat germ oil. The curves for the heated sera show exactly the behaviour that would be expected from the inactivation of the anti-tryptic factor. Another series of tests with serum to which had been added

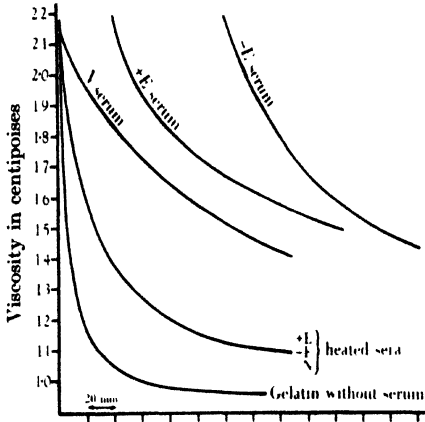


Fig. 4.

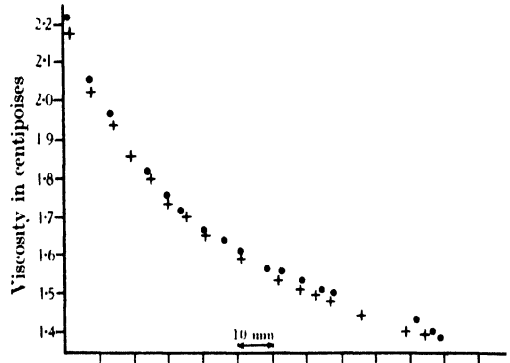


Fig. 5. • Normal serum + water.
+ Normal serum + 80 R.U. per ml. "Theelin".

8–80 R.U. of "Theelin" (Parke Davis) per ml. failed to reveal any suppression of tryptic activity as a result of the addition. The curves are superimposable (Fig. 5).

In all these experiments the course of the digestion was quite normal and there was not the slightest indication of any "all-or-none" effect such as Shute claims to have observed.

Although we are inclined to regard the different responses to the test which Shute describes as artefacts we prefer to express no opinion on the possible value of the test as an aid to diagnosis in gynaecological practice [cf. Cooper, 1939]. We are satisfied that vitamin E has no such influence as Shute describes on the "antiproteolytic power" of the serum of rats.

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CC. SPECTROSCOPIC CHANGES IN FATTY ACIDS

IV. ACIDS FROM SPECIMENS OF BUTTER FAT FROM COWS UNDER DIFFERENT NUTRITIONAL TREATMENTS

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THE presence in butter fat of acids showing spectroscopic absorption in the ultra-violet region was established by Gillam *et al.* [1931]. Booth *et al.* [1935] showed that the absorptive acids were present in greater concentration in summer than in winter butter fat, and that the absorption could not be due to oleic acid, or to polyethylenic acids in their usual form as present in seed fats. Dann *et al.* [1935] found that when fats containing polyethylenic acids are given to the cows they are converted from the spectroscopically "pro-absorptive"¹ into the "absorptive" state. This phenomenon was similar to that found to occur *in vitro* on the prolonged refluxing of cod liver oil with alcoholic KOH [Dann & Moore, 1933]. The purpose of these notes is to record data obtained upon three specimens of butter obtained from cows under different nutritional treatments.

EXPERIMENTAL

Fractionation of butter fat from cows at pasture. Spectroscopic data on the mixed butter acids from cows at pasture has already been given [Booth *et al.* 1935]. Through the kindness of Prof. T. P. Hilditch a series of fractions from the mixed liquid esters was made available (for details of the separation see Hilditch & Thompson [1936]). The spectroscopic absorption of the acids in the ultra-violet, as determined with a Hilger H 290 spectrophotometer, are given in Table I. A rapid increase of absorption with increasing I_2 values is evident.

¹ For definition see Dann *et al.* [1935], footnote, p. 139.

Table I. *Spectroscopic absorption of fractions of the mixed liquid esters of butter fat from cows at pasture*

No. of fraction	Wt. of fraction	Sap. equiv. (Hilditch)	i.v. (Hilditch)	$E_{1\text{ cm}}^{1\%}$ at 230 $m\mu$
1	31.23	227.6	33.5	5
2	6.37	280.3	71.8	9.7
3	8.99	286.1	80.1	12
4	10.46	291.7	87.1	13
5	11.14	293.4	91.9	20
6	8.85	296.0	94.0	21
7	10.6	297.6	96.3	23
8	6.19	298.7	96.9	38
Residue	6.46	325.5	99.7	84

Fractionation of butter fat from cows receiving cod liver oil. Spectroscopic data on the mixed butter acids from cows receiving cod liver oil have already been given by Dann *et al.* [1935]. Fractions from the mixed liquid esters of cows given cod liver oil were also made available by Prof. Hilditch. (See Hilditch & Thompson [1936].)

Table II. *Spectroscopic absorption of fractions of the mixed liquid esters of butter from cows receiving cod liver oil*

No. of fraction	Wt. of fraction	Sap. equiv. (Hilditch)	i.v. (Hilditch)	$E_{1\text{ cm}}^{1\%}$ at 230 $m\mu$
1	45.44	251.7	53.5	29
2	12.00	290.0	89.0	85
3	12.58	290.2	93.7	81
4	13.38	296.4	96.9	97
5	10.99	296.8	99.6	130
6	15.91	298.5	102.3	130
7	7.56	299.7	103.2	120
8	7.46	303.3	105.5	180
9	7.42	306.4	106.9	170
10	6.02	317.3	113.5	180
Residue	8.16	375.8	118.5	110

The maximum for absorption was always at about 230 $m\mu$, with no band at 270 $m\mu$. Further data were obtained on specimens which had been obtained by the refractionation of the fraction of lowest i.v. (53.5) in Table II. The usual association of intensity of absorption with i.v. is apparent in both primary and secondary fractionations.

Table III. *Refractionation of fraction 1 (Table II)*

No. of fraction	Wt. of fraction	Sap. equiv. (Hilditch)	i.v. (Hilditch)	$E_{1\text{ cm}}^{1\%}$ at 230 $m\mu$
1	3.04	182.7	10.5	2.1
2	4.37	215.5	17.2	2.6
3	5.45	238.0	33.3	3.3
4	5.89	252.5	37.1	6.7
5	7.85	265.9	55.9	20
6	5.36	280.4	77.1	40
7	4.97	288.9	89.7	65
8	4.57	296.9	99.3	86

Butter fat from cows receiving tung oil. Tung oil is unique among those fats already investigated in containing as its main constituent an acid (elaeostearic)

which shows intense spectroscopic absorption in the untreated oil. The absorption maximum is at about 270 $m\mu$.

Four cows which had been kept on a winter diet (hay 5 lb., mangels 36 lb., silage 18 lb., cubes¹ 4 lb. for each gallon of milk over 2 gallons) were divided into two pairs, the milk from each pair being combined. After a pre-experimental period of 8 days tung oil was added to the diet of one pair at the rate of 2 oz. twice daily, the other pair serving as controls. The tung oil was not readily ingested, even when mixed with bran and treacle. It was frequently refused at the morning feed, and was therefore given as a drench. It caused a decline in the yield of milk.

The spectroscopic changes produced are shown in Table IV. The feeding of tung oil caused a marked increase in absorption at 270 $m\mu$. Absorption at 230 $m\mu$ was not increased beyond the range of variation shown by the control group.

Table IV. *Effect of tung oil on the spectroscopic absorption of butter fat acids*

Date of beginning collection of milk	Valven and Valencia (controls)		Remarks	Pax and Orange (tung oil)		Remarks
	$E_{1\text{ cm.}}^{1\text{ cm.}}$			$E_{1\text{ cm.}}^{1\text{ cm.}}$		
	230 m μ	270 m μ		230 m μ	270 m μ	
14 Feb.	5.5	0.47	Pre-experimental period	7.9	0.39	Pre-experimental period
16 "	5.2	0.44	"	7.0	<0.3	"
18 "	8.3	0.32	"	4.7	0.55	"
20 "	3.8	0.29	"	6.3	0.28	"
22 "	8.2	<0.2	Controls	5.8	0.32	4 oz. tung oil daily
23 "	9.8	<0.2	"	8.1	1.24	"
24 "	8.5	<0.2	"	12	1.55	Oil partially refused
25 "	10	0.32	"	9.2	1.41	Drenched
1 Mar.	12	0.54	"	14	6.9	6 oz. tung oil daily
4 "	5.2	0.3	"	9.8	6.3	Tung oil stopped on 3rd
8 "	8.9	0.29	"	7.0	0.53	Cows recovered

$E_{1\text{ cm.}}^{1\text{ cm.}}$ at 270 m μ of the tung oil used = 1800

$E_{1\text{ cm}}^{1\text{ cm}}$ at 270 $m\mu$ of the tung oil used = 1800

DISCUSSION

The above results confirm the general principle that spectroscopic absorption, actual or potential, is concentrated in the more highly unsaturated fractions. They also support the previous finding that in butter fat the acids are present in the "absorptive" state. Attention may be drawn to the following additional points of interest.

Butter from cows at pasture. The presence of absorptive acids may be considered in relation to the evidence given in Part VI of this series [Moore, 1939, 1] that fatty acids of dried grass are present in the "pro-absorptive" state. A change in spectroscopic properties would therefore appear to take place in the cow, as previously found for fatty acids added to the diet [Dann *et al.* 1935].

Marine acids. The acids of cod liver oil, in common with those of most marine oils so far investigated, have the property of showing absorption at 270 $m\mu$ even more intensely than at 230 $m\mu$ as the result of a comparatively short (48 hr.) treatment with alcoholic KOH. The acids responsible for this absorption do not appear to be solid, as in the case of elaeostearic acid or of the acid absorbing in the same position which may be obtained from linseed oil by more prolonged treatment with KOH. It is remarkable that in the butter

¹ The cubes were composed of oil 4.1 %, albumin 16.4 %, carbohydrate 74.9 %, fibre 4.6 %.

fat the increased absorption caused by giving cod liver oil to the cow is situated at 230 $m\mu$ only, without any sign of a maximum at 270 $m\mu$, either before or after treatment of the butter acids with KOH. This must either imply that the acids absorbing at 270 $m\mu$ are not included in the butter fat, either through failure of absorption or rapid catabolism, or that they are in some way converted into acids absorbing at 230 $m\mu$.

Elaeostearic acid. The effect on the butter fat of giving tung oil was relatively small, suggesting as the most probable explanation that this acid is not well absorbed from the intestinal tract by the cow. The highest concentration reached in the butter fat as the result of feeding 6 oz. daily was about 0.5 %. The acid was apparently included in an unchanged state, and could be detected with certainty even in this low concentration through its very intense absorption. It is noteworthy that there was no change in the position of absorption maximum to 230 $m\mu$, as found when tung oil is fed to hens (Part V) [Moore, 1939, 2] or rats [Miller & Burr, 1937].

The cause of the failure of the acids of butter fat to form crystalline bromides. Green & Hilditch [1935] have found that the linoleic acid of butter fat does not yield a crystalline tetrabromide. Presumably the acid is present in a different isomeric form from that found in seed fats. This view is supported by the results obtained in the present work, since the linoleic acid of butter fat is spectroscopically in the "absorptive" state, which would not be expected to give crystalline bromides. The form present in butter, however, would appear to be different from that present in grass, having undergone change to the absorptive form in the cow, as stated above.

SUMMARY

1. Fractions of the mixed liquid fatty acids obtained by high vacuum distillation of the butter fat of cows at pasture showed spectroscopic absorption at 230 $m\mu$. The intensity of this absorption increased rapidly with the ascending I.V. of the fractions, in agreement with the conclusion that spectroscopic absorption is attributable to polyethylenic acids.

2. Similar results were obtained for the fractions of the liquid acids from the butter of cows given cod liver oil. According to the I.V., the content of polyethylenic acids was greater than that in the fractions from the pasture butters, and the spectroscopic absorption at 230 $m\mu$ was relatively more intense. No evidence was obtained of the transference to the milk of the acids present in cod liver oil "pro-absorptive" at 270 $m\mu$.

3. Tung oil was poorly tolerated by cows, but small doses resulted in the appearance of spectroscopic absorption at 270 $m\mu$ in the butter acids at an intensity suggesting the inclusion of about 0.5 % of unchanged elaeostearic acid. There was no evidence of the increased absorption at 230 $m\mu$ reported in the hen and rat when given tung oil.

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**CCI. SPECTROSCOPIC CHANGES IN
FATTY ACIDS**

**V. THE EFFECT OF THE DIETARY FAT ON
THE BODY FAT AND EGG FAT OF THE HEN**

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In Part I of this series [Moore, 1937] it was shown that while the polyethylenic acids of seed fats are usually in the spectroscopically "pro-absorptive" state, those present in animal fats may be either "pro-absorptive" or "absorptive". Thus in fishes and marine animals "pro-absorptive" polyethylenic acids have been found, whereas in land mammals, a part, at least, has been found in the "absorptive" state. The one bird fat investigated (duck) was found to contain "pro-absorptive" acids. It will be shown below that this finding also applies to the hen under normal conditions of nutrition. The results furnish evidence that divergences may exist between different species in their treatment of fatty acids.*

EXPERIMENTAL

Eggs were first collected by trap-nesting from hens which had been maintained since hatching on the same diet, which was designed to have a low content of fat (de-germed maize 40 parts, bran 15, yeast 5, dried skimmed milk 15, alfalfa 5, polished rice 20, mineral mixture 2.5). The egg fat was obtained by grinding up the yolks with anhydrous Na_2SO_4 and extracting for prolonged periods with ether. The acids were prepared by a brief saponification with alcoholic KOH, followed by the removal of unsaponifiable matter and acidification in the usual way. They were examined spectroscopically as so obtained and after refluxing for 48 hr. with alcoholic KOH.

Selected hens were then given the same diet but with the addition of sperm oil, linseed oil or tung oil. Eggs were collected and examined as before. The effect of the oils on the spectroscopic absorption of the egg and abdominal fats is shown in Tables I-V.

For convenience results are given to two figures, although the second is of doubtful significance if the first is high. Efforts were made to keep the conditions of refluxing constant but large differences were often observed between values which would be expected to be closely similar. The tendency of the added oils to increase absorption was, however, evident in all instances.

*Sperm oil.*¹ After eggs had been collected from a hen receiving the "low fat" diet an addition of 10% of sperm oil was made, over a period of 4 weeks. Eggs were collected at intervals.

Table I. *Spectroscopic absorption of the fatty acids of the eggs of hens receiving sperm oil*

Hen no.	Date	$E_{1\text{ cm.}}^{1\%}$ Short saponification (3 min.)		$E_{1\text{ cm.}}^{1\%}$ Long saponification (48 hr.)		Remarks
		230 m μ	270 m μ	230 m μ	270 m μ	
125	22. xi. 36	2.7	0.3	17	11	"Low fat" diet
	23. xi. 36	3.2	0.6	12	6.5	
	26. xi. 36	2.9	0.36	19	18	
	Means	2.9	0.42	16	12	
	17. i. 37	2.7	0.41	25	22	Sperm oil (10%) added to diet
4. ii. 37	2.4	0.37	33	33		
5. ii. 37	4.9	0.84	17	29		
8. ii. 37	5.1	0.72	22	28		
Means	3.8	0.59	24	28		
$E_{1\text{ cm.}}^{1\%}$ for fatty acids of sperm oil						
		10	<1	55	47	

The absorption of the acids was low when prepared by a short saponification. Refluxing with KOH increased the absorption, the intensity at 270 m μ having a mean value slightly greater than at 230 m μ .

Linseed oil. The experiment was conducted as above but with the addition to the diet of 10% of linseed oil for 11 days.

Table II. *Spectroscopic absorption of the fatty acids of the eggs of hens receiving linseed oil*

Hen no.	Date	$E_{1\text{ cm}}^{1\%}$ Short saponification (3 min.)		$E_{1\text{ cm}}^{1\%}$ Long saponification (48 hr.)		Remarks
		230 m μ	270 m μ	230 m μ	270 m μ	
375	2. ii. 37	2.1	0.30	26	20	"Low fat" diet
	4. ii. 37	3.3	0.39	23	18	
	5. ii. 37	2.1	0.51	29	25	
	Means	2.5	0.40	26	21	
	12. ii. 37	2.6	1.0	62	43	Linseed oil (10%) added to diet
15. ii. 37	2.9	0.36	96	63		
17. ii. 37	3.5	0.45	55	38		
Means	3.0	0.60	71	48		
$E_{1\text{ cm}}^{1\%}$ for fatty acids of linseed oil						
		2.5	—	250	90	

The absorption was again low when the acids were prepared by a short saponification, and was increased by refluxing with KOH.

¹ The oil was ordered as "whale oil", a description which is properly restricted to the oils of whales belonging to the *Balaena* and *Balaenoptera* genera. On examination after the experiment, it was found to have an unsaponifiable matter content of 35%. This high value is typical of sperm oil, i.e. the oil obtained from the sperm whale, *Physeter macrocephalus*, which contains a large proportion of the sperm wax.

Tung oil. Tung oil (5%) was added to the diet for 6 weeks.

Table III. *Spectroscopic absorption of the fatty acids of the eggs of hens receiving tung oil*

Hen no.	Date	$E_{1\text{ cm.}}^{1\%}$ Short saponification (3 min.)		$E_{1\text{ cm.}}^{1\%}$ Long saponification (48 hr.)		Remarks
		230 m μ	270 m μ	230 m μ	270 m μ	
1	22. xi. 36	2.5	0.19	33	20	"Low fat" diet
	25. xi. 36	2.9	0.21	11	4.9	
	Means	2.7	0.20	22	12	
	13. ii. 37	67	11	110	29	Tung oil (5%) added to diet
	14. ii. 37	52	8.5	120	49	
	21. ii. 37	90	15	120	32	
	Means	70	12	120	37	
43	19. ii. 37	89	19	113	37	Tung oil (5%) added to diet
		$E_{1\text{ cm.}}^{1\%}$ for fatty acids of tung oil				
		100	1500	—	—	

Fatty acids from the eggs obtained after giving tung oil differed from all others in showing intense preformed spectroscopic absorption. In surprising contrast with the position of absorption in the tung oil ($E_{1\text{ cm.}}^{1\%}$ at 270 m μ = 1650) the absorption maximum in the egg fats was at 230 m μ . Absorption at 270 m μ was only about one-seventh to one-fifth as intense as at 230 m μ .

The intensity of absorption at both positions was increased by refluxing with KOH. The amount of this increase was greater than that shown when the hen was not receiving tung oil (see Table V) suggesting that the tung oil contained small amounts of "pro-absorptive" acids in addition to the absorptive elaeostearic acid, the absorption of which has been found in previous experiments to be unaffected by refluxing.

In view of the unexpected nature of the result an egg from another hen receiving tung oil was also examined. Similar values were obtained.

Abdominal fats. After the collection of eggs the hens were killed, together with an additional hen still receiving the "low fat" diet; and specimens of the abdominal fat were taken. The experimental procedure was the same as that employed for the egg fats.

Table IV. *Spectroscopic absorption of acids from the abdominal fats of hens receiving a diet with and without added fats*

Hen no.	Diet	$E_{1\text{ cm.}}^{1\%}$ 2 min. saponification		$E_{1\text{ cm.}}^{1\%}$ 48 hr. refluxing with KOH	
		230 m μ	270 m μ	230 m μ	270 m μ
26	Free from added oil	2.5	<0.4	22	2.8
125	Sperm oil	4.1	<0.41	24	3.5
375	Linseed oil	4.8	<0.48	110	75
1	Tung oil	90	17	—	—

The changes in spectroscopic properties were generally similar to those observed in the egg fats. With fats from hens on the "low fat" and sperm oil diets, however, the absorption at 270 m μ after refluxing was much lower than in the egg fats.

DISCUSSION

The above results indicate that the hen normally has its polyethylenic acids present in the egg fat and abdominal fat in the spectroscopically "pro-absorptive" form. An exception to this rule occurs when tung oil has been given. The data on egg fats are summarized with the inclusion of I_2 values in Table V.

Table V. *Summary of data on the spectroscopic absorption of the eggs of hens on a diet with and without added fats*

Hen no.	Diet	I.V. of ingested oil	I.V. of egg fat acids	$E'_{1\text{ cm}}$ 2 min. KOH		$E'_{1\text{ cm}}$ 48 hr. KOH	
				230 $m\mu$	270 $m\mu$	230 $m\mu$	270 $m\mu$
125	Fat-free	—	80	2.9	0.42	16	12
375	"	—	—	2.5	0.40	26	21
1	"	—	—	2.7	0.20	22	12
			Means	2.7	0.34	21	15
125	Sperm oil	91	83	3.8	0.59	24	28
375	Linseed oil	179	106	3.0	0.60	71	48
1	Tung oil	169	84	70	12	120	37

Eggs from hens receiving sperm oil or linseed oil. The marine oils, as a class, contain fatty acids which have the property of showing absorption at 270 $m\mu$ of intensity equal to or greater than that at 230 $m\mu$ as the result of relatively short treatment with KOH. This property was found in the eggs of the hen receiving sperm oil. When linseed oil had been given the intensity of absorption after refluxing was considerably greater at 230 $m\mu$ than at 270 $m\mu$. In agreement with the greater degree of unsaturation of linseed oil the increases in absorption after refluxing were much greater in both positions than with sperm oil.

Eggs from hens receiving tung oil. The effect of feeding tung oil on the spectroscopic absorption of the acids of the egg and abdominal fats was striking. The same phenomenon of the change in absorption from 270 to 230 $m\mu$ has recently been reported by Miller & Burr [1937] who found that the feeding of tung oil to the rat caused the development of absorption at 230 $m\mu$ in the fat throughout the body. The most probable cause of the change is the reduction of the number of conjugated unsaturated linkages of elacostearic acid from 3 to 2, either by hydrogenation or less probably by oxidation with loss of part of the carbon chain. As shown in Part VI [Moore, 1939] catalytic hydrogenation causes a rapid decrease in absorption at 270 $m\mu$ accompanied by an increase at 230 $m\mu$, and partial saturation is therefore a plausible explanation of the change of absorption *in vivo*. It may be noted that the ratio of absorptions at 230 and 270 $m\mu$ was much higher in the egg acids than in the artificial mixture prepared by catalysis, suggesting a more selective mechanism in the treatment of elacostearic acid by the hen.

Comparison of the treatment of polyethylenic acids by the cow and the hen. It may be noted that the two animals differ in their treatment of polyethylenic acids in three directions. (1) In the cow the polyethylenic acids of the butter fat are present almost entirely in "absorptive" form. In the egg and abdominal fat of the hen, unless tung oil has been given, they are present in "pro-absorptive" form. (2) When marine acids are given the components responsible for absorption at 270 $m\mu$ cannot be traced in either "absorptive" or "pro-absorptive" form in the butter fat. In the egg fat of the hen they are present in "pro-absorptive"

form. (3) When tung oil is given to cows only a small percentage of apparently unchanged elaeostearic acid appears in the butter. With the hen the elaeostearic acid is apparently converted into an acid absorbing at 230 $m\mu$.

SUMMARY

1. Fatty acids from the egg and abdominal fats of hens given diets with no added oil, or with the inclusion of sperm oil or linseed oil contained polyethylenic acids in the "pro-absorptive" state.

2. The dietary fat had a marked effect on the amount of polyethylenic acids present in the egg fat as revealed by spectroscopic examination after refluxing with KOH. Similar effects were observed for the abdominal fat.

3. With sperm oil added to the diet the increases of absorption after refluxing with KOH were about equally great at 230 and 270 $m\mu$. With the diet free from fat, and with linseed oil included, the increase in absorption at 230 $m\mu$ was greater than at 270 $m\mu$.

4. The inclusion in the diet of tung oil which absorbs intensely at 270 $m\mu$ caused a marked increase in the preformed spectroscopic absorption at 230 $m\mu$ in the egg and the body fatty acids. This change in the position of the absorption maximum is probably to be ascribed to the loss of one of the conjugated unsaturated linkages of elaeostearic acid.

5. The above results suggest that there are marked differences in the treatment of polyethylenic acids by the cow and the hen.

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CCII. SPECTROSCOPIC CHANGES IN FATTY ACIDS

VI. GENERAL

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IN the course of the research described in the preceding papers of this series some interesting questions arose as side issues of the problems immediately under consideration. An account of additional evidence obtained in the investigation of these points may serve to throw further light on the general significance of spectroscopic changes in fatty acids.

EXPERIMENTAL

The effect of hydrogenation on the spectroscopic properties of the acids of tung oil. The very intense absorption shown by elaeostearic acid at $270\text{ m}\mu$ is presumably to be related to the presence of three conjugated unsaturated linkages. In view of the finding that in the hen and rat the administration of tung oil leads to the appearance of an acid absorbing at $230\text{ m}\mu$ it seemed desirable to see whether a similar change could be effected by chemical means. Hydrogenation to an extent sufficient to saturate one of the three linkages appeared to be the treatment most likely to produce the desired effect.

A sample of tung oil was kindly hydrogenated by Lever Brothers Ltd. The hydrogenation was done at 180° with 0.64 % of Crosfield's powdered catalyst. Since tung oil gives an abnormal I_2 value (164 instead of the theoretical value of about 246) the degree of saturation was estimated by the H_2 uptake, which during the early stages of the hydrogenation was much greater than would be calculated from the fall in i.v. The results obtained are given in Table I. H_2 to an extent of 2/6 saturation caused a fall in i.v. from 162 to 147. The spectroscopic absorption ($E_{1\%}^{1\text{cm}}$) at $270\text{ m}\mu$ fell from about 1400 to 250, but $E_{1\%}^{1\text{cm}}$ at $230\text{ m}\mu$ rose from 75 to 280. Further hydrogenation to 3/6 saturation with the i.v. at 103 caused a reduction in $E_{1\%}^{1\text{cm}}$ at $230\text{ m}\mu$ to 62, and at $270\text{ m}\mu$ to 6. Still further hydrogenation resulted in the spectroscopic absorption falling to negligible levels at both wave-lengths.

Attempts to fractionate the acids obtained by 2/6 saturation of the tung oil with a view to obtaining a product showing absorption only at $230\text{ m}\mu$ were unsuccessful. A solid fraction which could be removed after keeping the mixed acids in the cold showed absorption in both positions. A similar result was obtained with the liquid fraction, even after the residual solid acids had been crystallized out by keeping for 12 months in a refrigerator.

The change in absorption from 270 to $230\text{ m}\mu$ on the hydrogenation of tung oil has also been reported by Hulst [1935].

Table I. *Hydrogenation of tung oil*

Fraction of H ₂ absorption	Litres H ₂ per 100 g. calc.	Litres H ₂ per 100 g. found	Observed I.V.	$E_{1\text{ cm}}^{1\%}$ of mixed acids	
				230 m μ	270 m μ
Nil	0	0	164	75	1400
2/6	8.2	8.2	147.2	280	250
3/6	12.3	12.3	103	62	<6
4/6	16.4	16.4	59.9	9	<2
5/6	20.5	20.0	6.8	—	—
6/6	24.6	22	3.8	—	—

The difference between the theoretical and observed H₂ absorptions for the 6/6 saturated fraction is probably due to leaks in the gas meters, which become significant over long hardening periods.

Oxidation of the solid acid obtained by refluxing the acid of linseed oils with KOH (pseudo-elaeostearic acid). Two small specimens of the solid acid described in Part II [Moore, 1937] were sent to Prof. T. P. Hilditch, who kindly undertook a preliminary study of the products produced by oxidation with alkaline KMnO₄. The only dibasic acid formed in each case was sebacic acid. Small amounts of monobasic acids having, in impure condition, an apparent equivalent of about 106 were also obtained.

The spectroscopic properties of storage fat from rats receiving different dietary fats. Mixed intraperitoneal and subcutaneous fat was taken from rats which for 4 weeks had received diets containing 30% of coconut oil, lard, linseed oil and cod liver oil respectively. The fatty acids were examined spectroscopically before and after refluxing for 48 hr. with alcoholic KOH. Except in the specimen from rats given coconut oil considerable amounts of "pro-absorptive" acids were present.

Table II

Fatty acids from rats given 30% of	I.V. of fat (rat)	$E_{1\text{ cm}}^{1\%}$			
		2 min. saponification		48 hr. saponification	
		230 m μ	270 m μ	230 m μ	270 m μ
Coconut oil	37	10	0.6	12	1.7
Lard	65	5.5	0.4	17	4
Linseed oil	78	11	<1	50	20
Cod liver oil	79	7	0.5	50	50

The spectroscopic properties of the acids of dried grass. Preparation of acids. 500 g. dried grass, supplied by the kindness of Mr H. J. Page of Imperial Chemical Industries, were shaken five times with 1500 ml. ethyl alcohol. The extracts were combined, the alcohol evaporated off and the green residue saponified rapidly with alcoholic KOH. After dilution with water the soaps were extracted six times with ether, the extracts being discarded. The soaps were acidified, and extracted with ether. The ether extract, which was still bright green, was washed with dil. H₂SO₄ partially to decolorize it, washed with water, dried with Na₂SO₄ and given a preliminary treatment with norite charcoal. The ether was evaporated and the residue extracted with light petroleum (b.p. 50–60°). Much gummy material was left undissolved. The solution was again shaken with norite, which caused almost complete decoloration, the final colour on evaporation being very faintly yellow. About 90 mg. of acids were obtained. They partially solidified on cooling and had an odour suggestive of linseed cake.

Spectroscopic examination. The grass acids prepared as above had $E_{1\text{ cm}}^{1\%}$ at 230 m μ = 20. There was no maximum at 270 m μ . The I.V. (Rosenmund & Kuhnhehn method) was about 160, which is in reasonably good agreement with

the value of 170 found by Smith & Chibnall [1932]. When the spectroscopic absorption is compared with the i.v. it is apparent that not more than a small proportion of the polyethylenic acids was present in the "absorptive" form.

DISCUSSION

The chemical change underlying increased absorption in polyethylenic acids. It was suggested [Moore, 1937] that the most probable cause of increased absorption was the re-orientation of unsaturated linkages into conjugated positions. The production of sebacic acid, $\text{COOH}(\text{CH}_2)_8\text{COOH}$, from the solid absorptive acid obtained by refluxing the acids of linseed oil would suggest that the three unsaturated linkages have been changed from 9 : 10, 12 : 13, 15 : 16 to 10 : 11, 12 : 13, 14 : 15, thus producing a length of saturated chain sufficiently long for the production of sebacic acid. Working on the same acid in Burr's laboratory Kass [1938] has independently reached the conclusion that the first double bond has changed from the 9 : 10 to the 10 : 11 position. [See Kass *et al.* 1939.]

The effect of hydrogenation in reducing absorption at $270\text{ m}\mu$ and increasing adsorption at $230\text{ m}\mu$ agrees with the conclusion that absorption in the former position is attributable to three conjugated unsaturated linkages, in the latter to two conjugated unsaturated linkages.

The spectroscopic properties of the fatty acids in the rat. Differences were reported [Moore, 1937] in the spectroscopic properties of acids from various fats: vegetable fats and the fats of marine animals were found to have their polyethylenic acids in the "pro-absorptive" state, whereas those of land mammals were in the "absorptive" state. The results of the present work indicate that while differences in the spectroscopic properties of polyethylenic acids certainly exist between various animals, no consistent generalization can be made. Thus for acids from rats which had received large amounts of unsaturated dietary fat the spectroscopic properties were substantially those of the ingested acids in unchanged state. The spectroscopic absorption of the acids of the storage fats after feeding coconut oil was, however, greater than that of the acids of coconut oil, which are almost saturated and spectroscopically "non-absorptive", and was little affected by refluxing with KOH. It appears probable therefore that the former conclusion that land mammals have their polyethylenic acid in "absorptive" form may apply when the intake of polyethylenic acid is low. When the intake is high the acids may be laid down unchanged in the fat depots. When large amounts of a fat differing widely from the usual composition of the fat characteristic of the animal are ingested it is obvious that the properties of the latter may be readily obscured.

The spectroscopic absorption of grass acids. Since the acids of grass were found to be in the spectroscopically "pro-absorptive" form it is clear that absorptive acids found in the fats of herbivorous animals must be changed into this condition in the animal.

SUMMARY

1. Catalytic reduction of tung oil caused a change in the position of the absorption maximum of the mixed acids from 270 to $230\text{ m}\mu$, i.e. the same change which occurs *in vivo* when tung oil is fed to a hen or rat. This finding supports the conclusion that absorption in polyethylenic acids at $270\text{ m}\mu$ is caused by three conjugated unsaturated linkages, absorption at $230\text{ m}\mu$ by two such linkages.

2. Evidence has been obtained supporting the view that the change underlying increased absorption in polyethylenic acids when submitted to prolonged

refluxing with KOH is a re-orientation of the unsaturated linkages from unconjugated to conjugated positions.

3. When large amounts of "pro-absorptive" polyethylenic acids are included in the diet of the rat they are stored in the fat deposits in unchanged spectroscopic state. The predominance of absorptive acids found in the fats of some mammals, e.g. the cow, cannot therefore be made the basis of a general rule. The absorption of the acids from rats receiving an almost saturated fat (coconut oil) suggests however that when the intake of polyethylenic acids is low these are largely converted into the absorptive form.

4. Polyethylenic acids from dried grass were found to be in the "pro-absorptive" form. It appears therefore that "absorptive" acids present in the fats of herbivorous animals such as the cow must be converted in the animal.

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CCIII. THYROXINE AND HYPERVITAMINOSIS-A

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A CONSIDERABLE literature has developed suggesting that the thyroid gland may be specifically involved in the metabolism of vitamin A. An antagonism between thyroxine and carotene was first suggested by Euler & Klussman [1932], who found that superficially the two substances had opposite actions on the growth of rats deficient in vitamin A. Abelin [1933] reported a decrease of vitamin A in the livers of guinea-pigs receiving thyroxine. The administration of thyroxine reduced the amount of carotene in the blood of drakes [Parhon & Werner, 1935]. In human beings suffering from hyperthyroidism, the amount of carotene and vitamin A in the blood was found to be relatively low [Wendt, 1935, 1], comparable with the low amounts found in patients with deranged fat metabolism. Thyroxine administered to rats on a diet low in vitamin A accelerated the depletion of vitamin A reserves, as determined by the onset of ophthalmia [Sure & Buchanan, 1937, 2] or by the vaginal smear technique [Greaves & Schmidt, 1936]. Thyroidectomized rats developed symptoms more slowly. Fasold & Heidemann [1933] found that the milk of thyroidectomized goats was unusually yellow, suggesting failure in the conversion of carotene into vitamin A.

It has also been reported that thyroxine decreases the symptoms of hypervitaminosis-A, and that vitamin A likewise decreases the symptoms of hyperthyroidism [Fasold & Peters, 1932]. However, olive oil or sesame oil could replace vitamin A in counteracting thyrotoxicosis. Vitamin A reduced the basal metabolic rate of rats receiving excess thyroxine [Rappai & Rosenfeld, 1935; Abelin, 1935; Abelin *et al.* 1936; Logaras & Drummond, 1938], and the vitamin also reduced the basal metabolic rate of human patients suffering from hyperthyroidism [Wendt, 1935, 2] along with the amelioration of other symptoms [Wendt, 1936; Dietrich, 1936; Fasold, 1937]. When vitamin A was administered to a child suffering from hyperthyroidism Catel [1938], however, found that the lowered level of vitamin A in the blood was raised, but that the abnormal basal metabolic rate and other symptoms of the disease were not affected.

In the Reid Hunt reaction in mice, excess of vitamin A reduced the protective action of thyroxine against the toxicity of acetonitrile [Fleischmann & Kann, 1936]. In the development of salamander larvae or axolotls both vitamin A and carotene have been reported to counteract the normal effect of thyroxine in stimulating the metamorphosis of these organisms [Fleischmann & Kann, 1936; Rokhlina, 1936].

The administration of vitamin A is said to affect the amount of colloid in the thyroid gland [Sherwood *et al.* 1934] and histological changes in the gland have been attributed both to vitamin A deficiency [Mitzkewitsch, 1934; Coplan & Sampson, 1935] and to vitamin A excess [Votila, 1938]. Apparently vitamin A also counteracts the effects of the thyrotropic principle of the pituitary, since, when large amounts of the vitamin were given, those histological changes in the thyroid gland ordinarily elicited by the hormone failed to appear [Schneider, 1934; Fellingner & Hochstaedt, 1936]. Decreased stores of hepatic vitamin A were observed both in rats and guinea-pigs after the administration of the thyrotropic

hormone [Schneider & Wiedemann, 1934]. Logaras & Drummond [1938], however, found that the administration of thyroxine to rats caused increased liver reserves of vitamin A.

By many of the workers mentioned above the existence of a specific interrelationship between vitamin A or carotene and thyroxine has been accepted as an established fact. However, there is a certain amount of evidence which appears to contradict this view. For example, analysis of the livers of human beings suffering from "thyroid diseases" showed an increase in vitamin A content [Moore, 1937] rather than a decrease, as might be expected if a true antagonism existed between thyroxine and vitamin A. Four cases of exophthalmic goitre had reserves of 180, 300, 375 and 375 i.u. per g. of liver, as compared with the median reserve in accidental death of 220 i.u. per g. Wolff [1932] reported a similar increase in hepatic vitamin A in the same disease. (Mean for five cases of exophthalmic goitre 210 i.u. per g., for accidental death 147 i.u. per g.) Furthermore, hyperthyroidism is accompanied by excessive excretion of calcium [Parhon, 1912; Heath *et al.* 1925; Aub *et al.* 1929], and in hypervitaminosis-A there is also a negative calcium balance [Harris, 1933] which proceeds until the bones are weakened to such an extent that spontaneous fractures occur [Bomskov & Seeman, 1933; Collazo & Rodriguez, 1933; Davies & Moore, 1934]. It is difficult to visualize a true antagonism between two agents producing in this instance similar physiological responses.

Finally, several groups of workers [Cowgill & Palmieri, 1933; Sure & Buchanan, 1937, 1; Drill & Sherwood, 1938; Peters & Rossiter, 1939] have shown that large amounts of vitamin B₁ protect rats against loss in weight caused by excessive doses of thyroxine.

These findings at least indicate that caution is necessary before the interrelation between vitamin A and thyroxine is regarded as specific. The experiments described below were done to test the question of the interrelationship of vitamin A and thyroxine when both are given in doses so large as to be toxic, but not rapidly fatal. Under these conditions no interrelation could be established.

EXPERIMENTAL

(i) *The effect of excessive amounts of thyroxine and high vitamin A alone and combined in adult rats*

The effect of large doses of thyroxine was studied in rats receiving excessive amounts of vitamin A. In the first experiments adult stock female rats, approximately one year old, were used. They had received a mixed diet rich in vitamin A and carotene, and at the commencement of the experiment had very high reserves of vitamin A (*ca.* 30,000 i.u.). They were divided into four groups of 8 rats each, and were fed on a basal ration low in vitamin A previously used in this laboratory [Davies & Moore, 1935]. The dry ingredients consisted of "light white" casein 200, sugar 450, salt mixture 50 and yeast 125 (total 825). 82 parts of the dry ration were mixed with 30 parts of an oily solution containing 298 g. of coconut oil and 2 g. of radiostol (vitamin D). The high fat diet was used to facilitate the absorption of vitamin A. The rats were grouped as follows.

Group I received the low-vitamin A diet plus an excess of vitamin A. The vitamin was given as a concentrate from halibut liver oil, prepared by the British Drug Houses, Ltd. It was dark brown, viscous, and smelled of ionone. The absorption band at 328 m μ was narrow and intense, $E_{2\%}^{1\text{cm}}$ = 732. If the absorption of pure vitamin A, $E_{2\%}^{1\text{cm}}$ = 1800 [Mead, 1938] our concentrate contained 40% of vitamin A by weight. For administration the concentrate was diluted

with 4 parts of arachis oil and fed by dropper, each rat receiving 4 drops daily. Since each drop weighed 25 mg. the rats received $25 \times 4 \times 20\% = 20$ mg. of concentrate daily, equivalent to 8 mg. of vitamin A. Solutions of the concentrate were made up weekly and were stored in a refrigerator. For our purpose it was not feasible to feed the vitamin A mixed in the basal ration, because of the inequality of food consumption between individuals and between groups.

Group II received the basal diet plus 8 mg. of vitamin A daily plus 0.6 mg. of thyroxine injected subcutaneously three times per week. The thyroxine (Hoffmann-La Roche) was dissolved in *N*/100 NaOH, neutralized with a few drops of *N*/100 HCl until the first appearance of cloudiness in the cold. It was diluted to a concentration of 2 mg. per ml. The solutions were made up fresh each week; they were stored in the cold and were warmed to 37° before injection.

Group III received the basal diet only.

Group IV received the basal diet plus thyroxine.

The basal ration was fed *ad libitum* in all cases, and the food intake of the various groups was recorded.

Results. All animals receiving either thyroxine or excess of vitamin A, or both, showed an immediate decrease in weight (Fig. 1) and a decreased food intake (Table I). There was no evidence that the thyroxine counteracted the effects of hypervitaminosis-A, or vice versa. Animals receiving thyroxine plus high amounts of vitamin A showed the greatest decrease in food intake of all groups and the most rapid loss in weight. The effects of excess of vitamin A and of excess of thyroxine therefore appeared to be additive rather than antagonistic in this experiment.

Symptoms of hypervitaminosis-A appeared in Group I (vitamin A alone) and Group II (vitamin A with thyroxine) between the fifth and tenth days of administration of the vitamin concentrate, confirming previous observations [Drigalski, 1933; Davies & Moore, 1934]. The animals were limp and emaciated. Their hair was rough and their posterior parts were smeared with urine. They sat in a hunched position. The eyes were swollen and in a few cases encrusted. There was loss of hair about the mouths, which were sore and inflamed, as if the vitamin concentrate were irritating. Four out of 6 rats in Group II (vitamin A with thyroxine) died during the first 9 days. The remainder died on the seventeenth and twenty-eighth days. No rats died in any of the other groups. Rats receiving the high vitamin A diet showed symptoms of hypervitaminosis throughout the experiment, but survived. Rats receiving the basal diet were normal in every way; rats receiving the low vitamin A diet plus thyroxine showed no gross symptoms other than a steady continuous decrease in weight.

Four animals in Group I (vitamin A) were killed on the tenth day, and their livers were found to contain from 60,000 to 150,000 I.U. of vitamin A as measured by the Carr & Price [1926] test. The livers of animals of Group II, which had died from the combined effects of thyroxine and hypervitaminosis-A, contained similar amounts of vitamin A, the values ranging from 60,000 to 180,000 I.U.

There was, therefore, no significant difference in the vitamin A contents of the two groups of animals. Thyroxine failed to decrease the amount of vitamin A stored in the liver under these conditions. Rats which survived for more than 30 days on the high vitamin A diet showed from 150,000 to 360,000 I.U. per liver.

A curious effect of thyroxine on the food intake of rats was noted—both in the presence and absence of excess of vitamin A. The injection of thyroxine markedly decreased the food intake for the first 10 days of administration, after which there was a sudden increase in food intake as compared with controls on the same diet (Table I). These results have been duplicated repeatedly. They clarify

certain inconsistencies in the literature, inasmuch as thyroid has been reported both to increase [Schafer, 1912; Hewitt, 1914, 2] and to decrease food intake [Hewitt, 1914, 1; Kojima, 1917].

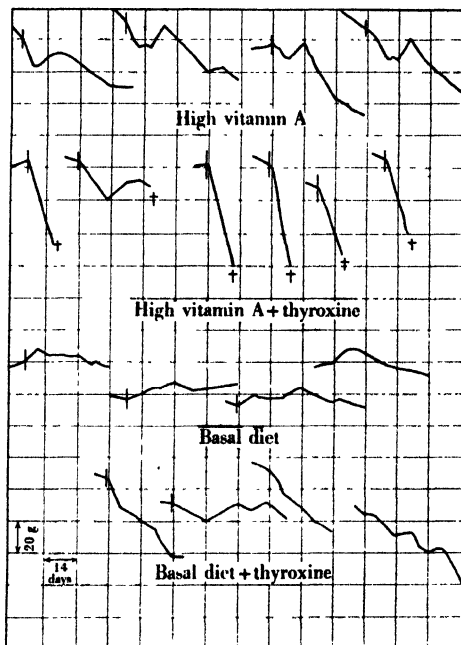


Fig. 1. Exp. 1. The additive action of thyroxine and excess vitamin A. Effects of excessive vitamin A and thyroxine separately and combined on the body weight of rats. Curves for rats in Group 1, which were killed after 10 days for vitamin A determinations, are not shown.

Table I. *The effect of excess thyroxine and vitamin A upon the food intake of rats*

	Food intake per rat per day (g.)			
	I High-vitamin A diet	II High-vitamin A + thyroxine	III Control diet	IV Control diet + thyroxine
First 5 days	3.8	1.9	9.0	5.3
Second 5 days	6.4	3.0*	7.0	4.6
Third 5 days	7.5	—	6.5	12.6
Fourth 5 days	4.9	—	5.8	11.5
Fifth 5 days	6.7	—	5.5	12.2
Sixth 5 days	6.0	—	6.8	13.8

* Only 2 out of 6 alive.

(ii) *Thyroxine and high vitamin A in growing rats*

The failure to demonstrate an antagonism between thyroxine and excess of vitamin A in adult rats led us to attempt similar experiments with young, growing animals, similar to those used by Fasold & Peters [1932]. Young rats, 4 weeks of age, 50–60 g. in weight, were given 2 drops daily of the vitamin A solution as before (=10 mg. B.D.H. concentrate daily=4 mg. vitamin A per rat per day). The basal diet was the same high fat ration used in the preceding experiment. Group food intakes were recorded.

Five rats received the high vitamin A diet alone; 5 received the high vitamin A diet plus thyroxine. A third group received the basal low vitamin A ration plus

0.2 mg. thyroxine daily, the dose being increased by 0.2 mg. daily according to the scheme of Fasold & Peters until a level of 1.0 mg. per day was reached. The fourth group received the low-vitamin A ration plus injections of water.

The animals receiving the high vitamin A diet only showed some indications of toxic symptoms. Within one week their fur became rough and matted; after 3 weeks the mouths of the animals became sore, growth ceased and a loss in weight followed. All animals in this group survived for 6 weeks, when the experiment was terminated.

Animals receiving the high vitamin A diet plus thyroxine, according to Fasold & Peters, stopped growing after the first week and died between the fourteenth and nineteenth days of the experiment. Instead of protecting the animals against the harmful effects of the vitamin, as claimed, the administration of thyroxine hastened death.

The animals receiving the thyroxine survived the 6 weeks of the experiment. Their rate of growth, however, was less than that of the rats receiving injections of water; they averaged 120 g. in weight at the end of the experiment, compared with 146 g. for the control animals. As with the adult animals, the administration of thyroxine caused an immediate decrease in food intake, followed after one week by a marked increase in food intake as compared with the control group.

DISCUSSION

The evidence which has been advanced to show a relationship between thyroxine and vitamin A has been derived from a field too wide to permit of comprehensive discussion in a paper dealing with only one aspect of the problem. There would seem ample grounds, however, for believing that in the abnormal metabolic states caused by inadequacy or excess of thyroxine, corresponding abnormalities occur in the metabolism of carotene and vitamin A. It is a more open question how far these abnormalities imply a specific relationship between the two substances. Disturbances in vitamin A metabolism occur in other diseases, e.g. in chronic nephritis, in which low liver reserves are usually found, and probably in diabetes, in which the reserves are high [Moore, 1937]. Stringent proof would appear to be necessary before a specific and constant, as opposed to an accidental and occasional, relationship between vitamin A and thyroxine is assumed.

One of the most suggestive points in favour of a specific relationship has been the reported ability of thyroxine to counteract the ill effects of excess of vitamin A, and vice versa, which has been re-investigated with negative results in the present work. It is possible that our failure to observe this effect may have been due to some minor difference between our experimental conditions and those adopted by Fasold & Peters [1932]. In a complicated condition in which the various body tissues come under the toxic influence of two distinct agents it appears that effects might be additive or subtractive according to the nature of the tissues and the dosages employed. The experiments of Logaras & Drummond [1938] in which beneficial effects were produced by large but non-toxic allowances of vitamin A in rats given moderately excessive amounts of thyroxine, represent a limited confirmation of the claims of Fasold & Peters. They do not deal, however, with the question of the neutralization of the excessive doses of vitamin A and thyroxine which given separately would both be highly toxic. While, therefore, our experiments do not rule out the possibility that under some conditions vitamin A and thyroxine may neutralize each other, they suggest that such antagonism is neither as specific nor as universal as hitherto supposed.

The effect of thyroxine in temporarily decreasing and then increasing the food intake is readily explicable if it is assumed that the action of this hormone is

primarily confined to the catabolic side of metabolism. The increase in food intake is presumably a secondary phenomenon and is probably delayed until the organism has had time to adjust itself to the increased metabolic rate.

SUMMARY

When injected into rats receiving excess of vitamin A, thyroxine failed to counteract the effect of hypervitaminosis. Animals receiving both thyroxine and excess of vitamin A ate less, lost weight more rapidly, and died earlier than those receiving either agent alone. The injection of thyroxine produced a temporary decrease in food intake, followed by a marked increase. No evidence was obtained for a specific antagonism between thyroxine and vitamin A.

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CCIV. THE VITAMIN A CONTENT OF CHEESE

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THE vitamin A content of cheese was investigated by Morgan [1926] who found that samples of American Cheddar and Limburger restored growth and cured xerophthalmia in rats when given in doses of 1 g., later reduced to 0.5 g. In the case of a Swiss cheese 1.0 g. daily was required.

More recently Coward & Morgan [1935], in a comprehensive study of the amounts of vitamins A and D in many common foodstuffs, have reported a potency of 55 I.U. per g. for one specimen of English Cheddar cheese. This value seems high compared with values quoted by other workers for butter fat. Thus Booth *et al.* [1934] assess the vitamin A contents of winter, autumn and summer butter fats at 40, 65 and 85 I.U. per g. respectively. Assuming the fat of cheese to have a vitamin A potency equal to that of butter fat, and taking the fat content of Cheddar cheese as 26.8% [Hutchison & Mottram, 1933], these values for butter fat would indicate values between 11 and 23 I.U. per g. for the cheese, according to whether it were made from winter or summer milk.

The high value given by Coward & Morgan might therefore imply either that the specimen of cheese examined had been prepared from milk of exceptionally high potency, or alternatively that cheese has a vitamin A activity disproportionately greater than its fat content. This latter possibility might arise either through the vitamin being concentrated in some way in the protein fraction of the milk, or through a better absorption of the vitamin from the intestinal tract. In view of the interest of these implications, and of the importance of cheese as an article of diet, we have carried out an independent test of its vitamin A activity.

EXPERIMENTAL

Colorimetric estimations. A sample of English Cheddar cheese was purchased locally and examined for vitamin A and carotene by colorimetric methods. Since these substances were present in very small concentrations the results obtained were of a low degree of accuracy, being useful only as a rough indication of the order of activity to be expected in biological tests. Vitamin A, as determined by the SbCl_3 method applied to the unsaponifiable residue of the cheese fat (extracted by digestion with alkali), was present to the extent of about 5 B.U. per g., equivalent to about 3 I.U. per g. [Moore, 1937]. The carotene content determined by the tintometer, with the use of a reference curve, was about 3.5 μg . (6 I.U.) per g. expressed as the gross yellow colour of the unsaponified cheese fat, or 1.8 μg . (3 I.U.) per g. expressed as the gross yellow colour of the non-saponifiable matter. The first of these values may be high through the inclusion of yellow or brown substances other than carotene, the second low on account of the loss of pigment during manipulation.

When the values obtained for vitamin A and carotene are combined the total vitamin A activity of this cheese would appear to be about 6–9 I.U. per g. Since

the fat content of the cheese was found to be about 33 % these values would be equivalent to 18–27 I.U. per g. of fat.

An attempt to confirm the vitamin A content by the determination of absorption at 328 m μ was unsuccessful on account of the intensity of the general absorption at this position.

Biological tests. Young albino rats of body weights 39–66 g. were given a basal diet of caseinogen (alcohol-extracted) 20 %, cane sugar 60 %, arachis oil 15 %, salt mixture 5 %, with dried yeast 10 %, Radiostol (vitamin D) 1 drop per rat per week. Growth ceased after 37–40 days. To one group of two male and two female rats a solution of the International Standard carotene was given at a level (12.8 I.U. every 4 days) found by previous experience to give fairly rapid growth. To two other similar groups cheese was given at the levels of 250 mg. and 1750 mg. per 4 days. These levels were chosen so as to be equivalent to the dosage of carotene on the basis of cheese containing 35 I.U. per g. and 7.5 I.U. per g. respectively. (On recalculation after the experiment these doses were found to be slightly out of adjustment, actually corresponding to 13.7 and 13.1 I.U. per 4 days.) To save time in weighing, the doses of cheese were given as portions of uniform diameter cut by a cork borer, the lengths necessary for the dose being found by experiment. They were consumed rapidly by the rats. The growth responses were as follows.

Daily addendum	Calculated equivalent in I.U. per day taking cheese = 55 I.U. per g.	Calculated equivalent in I.U. per day taking cheese = 7.5 I.U. per g.	Mean wt. increase per rat per day (groups of 2 ♂ and 2 ♀ rats g.)
3.2 I.U. of standard carotene	—	—	1.24
62.5 mg. of Cheddar cheese	3.4	0.47	0.69
437 mg. of Cheddar cheese	24	3.3	1.36

Colorimetric tests on other types of cheese. Colorimetric estimations were carried out on several other types of cheese. Amounts of vitamin of the same order as those found in Cheddar cheese were found in other cheeses made from whole milk (Camembert, Cheshire, Empire red, Empire white, Gruyère, Stilton). Smaller amounts were found in cheeses of lower fat content (Dutch Edam and Danish blue).

DISCUSSION

In the above experiments nearly equal growth responses (1.36 and 1.24 g. per day) were obtained when English Cheddar cheese and the International Standard carotene were given as sources of vitamin A in equivalent doses, calculated on the assumption that the cheese contained vitamin A and carotene in the amounts found by colorimetric methods. When the equivalent doses were calculated on the basis of Coward & Morgan's value of 55 I.U. per g. the growth response with cheese (0.69 g.) was much lower than that with the carotene. It is clear that the difference between the activity found in our experiment and that reported by Coward might be accounted for if her cheese had been made from an exceptionally rich milk. No data on this point or on the activity of the milk from which our own sample of cheese was made are available. Our own experiments however indicate that the vitamin A potency of the cheese examined by us was certainly not greater than would have been anticipated from its content of milk fat. We have therefore no evidence of any hidden form of activity in cheese, or of exceptionally efficient utilization of the vitamin.

SUMMARY

English Cheddar cheese was found in biological tests to have a vitamin A potency of an order (*ca.* 7.5 I.U. per g.) that would be expected from its milk fat content, and from the result of colorimetric estimations of vitamin A and carotene. Colorimetric determinations on other full-milk cheeses gave similar results. Lower values were found for cheeses of lower fat content.

Our thanks are due to Dr L. J. Harris for his valuable criticism.

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CCV. THE VITAMIN A CONTENT OF "LIGHT WHITE" CASEIN

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THE presence of vitamin A in specimens of casein not subjected to treatment for effecting its removal or destruction has been assumed from the early days of research on vitamins. "Light white" casein (sodium caseinate), a form of casein much used in nutritional experiments, was examined as a source of vitamin A for rats by Culhane [1933]. She found that its use as the protein constituent of a basal diet for rats in place of inactivated casein prevented or delayed xerophthalmia and prolonged the survival period. Without further supplements of vitamin A, however, the rats eventually stopped growing and showed signs of deficiency. Culhane calculated that "light white" casein possesses 1/200th of the activity of cod liver oil, and that its inclusion in the diet at the level of 20 % was equivalent to a daily dose of 0.05 mg. of carotene. These conclusions would appear to be based on unduly low estimates of the activities of carotene and cod liver oil.

As far as we are aware vitamin A has not hitherto been detected in casein by chemical means. The object of the experiments described below has been to confirm the presence of vitamin A by this means, to arrive at a rough approximation of the amount present and to observe whether or not the vitamin is present in any unusual form or state of combination.

EXPERIMENTAL

Specimens of "light white" casein (40–300 g.) were extracted under different conditions as shown in Table I. The solvents were usually employed at the rate of 5 ml. per g. of caseinogen. Ether and ethylene dichloride were used cold. Ethyl alcohol was used at boiling point by refluxing, usually for periods of 2 hr.

Amounts of fat obtained. With cold ether or ethylene dichloride the amounts of fat obtained were much smaller than with hot alcohol. Moreover, the fat appeared to dissolve in two distinct fractions. In experiments in which successive extractions were made a yield of about 0.4 % of fat was usually obtained in the first extraction in the cold. In a second extraction in the cold the yield was very small. A further yield of more than 1 % was, however, obtainable by using hot alcohol.

Colorimetric estimations. The fatty extracts were saponified and the non-saponifiable residues extracted in the usual way. The determination of yellow coloration in the untreated extract and of the blue coloration after the adding of SbCl_3 was made with a Lovibond tintometer. With the small amounts of fatty extract available, and with only a low concentration of vitamin A present, highly accurate results were not to be expected.

With the extracts obtained with ether and ethylene dichloride blue colours were never given in the SbCl_3 tests. In view of the very small amounts of material available it is doubtful whether a complete absence of vitamin A from these fractions is to be inferred. With the extracts obtained with hot alcohol blue colours were always given. In agreement with experience with similar extracts from butter fat the blue colours faded very rapidly; accurate matching in the tintometer was therefore difficult. With one of the extracts richest in vitamin A a faint absorption band at $620 \text{ m}\mu$ was observed.

Table I. *The colorimetric examination of fat extracted from specimens of "light white" casein*

Specimen	No. of exp.	Solvent	% fat obtained	Lovibond units per 100 g. casein		Remarks
				Y.U.	B.U.	
A	1	Ethylene dichloride, 2 extractions	0.5	12.5	0	Casein, after 2 more extractions, + in biological tests
	2	Ethyl alcohol, hot, 2 extractions	2.0	50	50	
B	3	Ether	0.45	28	0	Extracted casein, + in biological tests
	4	Ether, 1st extraction	0.4	10	0	
		Ether, 2nd extraction	0.09	2	0	
		Total	0.49	12	0	
	5	Alcohol, hot, 1st extraction	1.7	82	27	Extracted casein, - in biological tests
		Alcohol, hot, 2nd extraction	0.17	3	2	
		Total	1.87	85	—	
	6	Ether, 1st extraction	0.39			
		Ether, 2nd extraction	0.025			
		Alcohol, hot, 3rd extraction	1.34			
		Total	1.76			
	7	Ether, 1st extraction	0.43			
		Ether, 2nd extraction	0.028			
		Alcohol, hot, 3rd extraction	1.14	50	23	
		Total	1.60			
C	8	Alcohol, hot	—	90	100	620 $\text{m}\mu$ absorption band observed

All the extracts were coloured yellow. The yellow/blue ratio was so high in some instances as to suggest that the blue colour might be mainly derived from carotene, but in others (Exps. 2 and 8, Table I) lower ratios indicated the presence of vitamin A. In view of the possibility of the presence of yellow pigments other than carotenoids it does not seem safe to assume the absence of vitamin A, even from the extracts with high yellow/blue ratios.

If we make the assumption that all the yellow pigment of the extracts was carotene the total vitamin A activity of the two richest extracts (Exps. 5 and 8, Table I) may be calculated as about 80 and 120 I.U. per 100 g. respectively. The value of 1 I.U. per g. would seem a reasonable approximation for a typical casein. Taking the fat content at the round figure of 2%, the vitamin A of the fat amounts to 50 I.U. per g. This value lies within the range given by Booth *et al.* [1934] for butter fat. A rat eating daily 10 g. of a basal diet containing 20% of caseinogen would receive 2 I.U. of vitamin A activity.

Biological tests. In our main biological experiments young rats were given a diet deficient in vitamin A (caseinogen 20%, sugar 60%, arachis oil 15%,

salt mixture 5% and dried yeast 10%, radiostol (vitamin D) 1 drop per rat per week. The caseinogen was supplied by Glaxo Laboratories, Ltd. It was of the "acid type" and had been extracted with hot alcohol. After about 6 weeks a growth plateau was attained. Groups of rats were then given the same basal diet but with the Glaxo caseinogen replaced by (a) untreated "light white" casein, (b) "light white" casein which had been extracted twice with ether (Exp. 4, Table I), (c) "light white" casein which had been extracted twice with hot alcohol (Exp. 5, Table I). The growth responses observed over a period of 6 weeks are given in Table II.

Table II. *Biological tests on untreated and extracted "light white" casein*

	Rat no. and sex	Caseinogen used	Wt. increase and condition after 6 weeks
Group A	1 ♂	Untreated "light white" caseinogen	In good condition. Gained 33 g.
	2 ♂	" " "	In fair condition. Wt. increased by 3 g.
	3 ♀	" " "	In good condition. Wt. increased by 19 g.
	4 ♀	" " "	In good condition. Wt. increased by 19 g.
Group B	5 ♂	"Light white" caseinogen extracted with cold ether	In good condition. Gained 24 g.
	6 ♂	" " "	Died after 17 days. Lost wt. rapidly during the last week
	7 ♀	" " "	In fair condition. Wt. maintained
	8 ♀	" " "	In good condition. Wt. increased by 16 g.
Group C	9 ♂	"Light white" caseinogen extracted with hot alcohol	Died after 14 days. Lost 47 g.
	10 ♂	" " "	Died after 10 days. Lost 21 g.
	11 ♀	" " "	Died after 38 days. Lost 58 g., xerophthalmia
	12 ♀	" " "	Died after 20 days. Lost 44 g., slight xerophthalmia
	13 ♀	" " "	Died after 13 days. Lost 29 g.

It will be seen that all the rats survived, with slow growth, when untreated "light white" casein was included. With "light white" casein extracted with ether 1 out of the 4 rats died, 1 maintained its weight and 2 showed slow growth, indicating a partial loss of activity from the caseinogen. With "light white" casein extracted with hot alcohol all 5 rats in the group lost weight rapidly and died.

In preliminary experiments another sample of untreated "light white" casein promoted slow growth in 2 rats over periods of several weeks, after which growth stopped, with decline and death in one animal. The same casein after 4 extractions with ethylene dichloride (Exp. 1, Table I) caused restoration of growth for about 4 weeks, after which constant weight was maintained.

In our experience about 1-2 I.U. of vitamin A are usually necessary to promote slow growth in rats. The results of these biological experiments indicate, therefore, that "light white" casein before extraction has a biological activity in good agreement with the results of the colorimetric determinations, and with the general inference that the fat present has an activity approximately equal to that of butter fat.

SUMMARY

1. "Light white" casein contained enough vitamin A to promote slow growth in rats depleted of the vitamin when included in the diet at the level of 20 %.

2. When specimens of the casein were extracted with hot alcohol the extracts were coloured yellow and gave blue colorations when treated with the SbCl_3 reagent, indicating that carotene and vitamin A were present in amounts consistent with the biological activity. The value of 1 I.U. per g. may be taken as the approximate vitamin A content of typical casein.

3. Ether and ethylene dichloride, used in the cold, extracted only a small fraction of the fat present in "light white" casein, and the SbCl_3 reaction could not be demonstrated in the extracts. The vitamin A activity of the casein was at least partially retained.

Our thanks are due to Dr L. J. Harris for his valuable criticism.

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CCVI. THE RETENTION OF CALCIUM BY THE RAT IN THE PRESENCE AND IN THE ABSENCE OF VITAMIN C

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It is usually considered that the rat does not require vitamin C for normal nutrition. Recently, however, Fairbanks & Mitchell [1937] have reported preliminary experiments showing that the retention of Ca, fed at a sub-optimal level and derived from spray-dried skim milk, could be improved by the daily administration of 1 mg. of crystalline ascorbic acid. Our experience [Henry & Kon, 1939] did not support these findings and the present work was undertaken to put the problem to direct test.

EXPERIMENTAL

A basal diet containing Ca in inorganic form was used in preference to a skim-milk diet, as dried milk may contain appreciable quantities of vitamin C [Henry *et al.* 1939]. Diet 92 of Henry & Kon [1939] was used; it contained 0.1561 % Ca, 0.2413 % P, 10.81 % moisture, and only traces of vitamin C.

Eight sets of 3 litter-mate male rats aged 22–25 days and weighing 45–64 g. were placed in metabolic cages made of glass and stainless steel. The experimental technique was as described in detail by Henry & Kon [1937]. All animals were given the basal diet and the food intake was equalized within groups of 3.

An Osborne & Wakeman [1919] yeast extract was given as a source of the vitamin B complex. This extract was fortified with vitamin B₁ by the addition of 10 mg. of aneurin to about 300 ml. of the extract. The animals received 0.4 ml. of this extract daily. It contained 0.0328 g. Ca and 1.1345 g. P per 100 ml. Allowance was made for this in calculating the intakes of Ca and P. Two drops of cod liver oil were given daily as a source of vitamins A and D. In each group at the end of 3 days the pair of rats nearest in weight was kept and the third rat was killed for analysis. The rats were allocated by toss of coin to the control group or to the vitamin C group. In the latter group the animals were given by pipette 2 mg. of ascorbic acid daily as a freshly prepared aqueous solution. The control group received similarly 2 mg. daily of glucose. The diet intake was equalized within pairs.

The experiment lasted 5 weeks and the cages were washed once a week. At the end of the experiment the animals were killed by coal gas and the carcasses analysed for Ca and P. In the course of the experiment the animals shed a considerable amount of hair. This was collected separately for each group and analysed for Ca and P. No Ca was found and the total loss of P in this way amounted to only 0.2 mg. per rat.

Table I. *Growth and Ca and P balances of the rats*

Rat No.	Wt. of rat (g.)		Diet intake (g.)	Ca intake (g.)*	Ca excretion (g.)		Ca balance (g.)	% Ca retention	P intake (g.)†	P excretion (g.)		P balance (g.)	% P retention	Wt. of faeces (g.)
	Initial	Final			Urine	Faeces				Urine	Faeces			
1	62	170	366.43	0.5766	0.0124	0.0561	Control group 0.5081	88.1	1.0430	0.4418	0.1365	0.4647	44.6	8.63
3	68	168	382.46	0.6016	0.0055	0.0284	0.5677	94.4	1.0817	0.4751	0.1136	0.4930	45.6	8.16
5	62	164	353.79	0.5569	0.0077	0.0380	0.5112	91.8	1.0125	0.4670	0.0986	0.4469	44.1	6.82
7	58	167	375.96	0.5915	0.0046	0.0295	0.5574	94.2	1.0690	0.4838	0.0908	0.4914	46.1	6.50
9	54	138	305.75	0.4819	0.0059	0.0317	0.4443	92.2	0.8966	0.4275	0.0796	0.3895	43.4	5.28
11	53	158	392.68	0.6176	0.0054	0.0237	0.5885	95.3	1.1063	0.5176	0.0824	0.5063	45.8	6.02
13	58	145	332.90	0.5243	0.0091	0.0364	0.4788	91.3	0.9621	0.4381	0.1104	0.4136	43.0	7.71
15	55	137	311.00	0.4901	0.0043	0.0528	0.4330	88.3	0.9092	0.3945	0.1387	0.3760	41.4	8.01
Mean	58.8	155.9	352.62	0.5551	0.0069	0.0371	0.5111	91.05	1.0097	0.4557	0.1063	0.4477	44.25	7.14
Vitamin C group														
2	62	172	366.26	0.5763	0.0136	0.0319	0.5308	92.1	1.0426	0.4899	0.0832	0.4695	45.0	5.70
4	68	160	382.33	0.6014	0.0061	0.0472	0.5481	91.1	1.0814	0.5546	0.1313	0.3955	36.6	8.46
6	61	151	355.99	0.5603	0.0035	0.0300	0.5208	93.0	1.0178	0.4636	0.1113	0.4429	43.5	7.50
8	57	167	375.58	0.5909	0.0029	0.0403	0.5477	92.7	1.0651	0.4840	0.0875	0.4936	46.3	5.71
10	54	131	305.94	0.4822	0.0056	0.0427	0.4339	90.0	0.8970	0.4300	0.1008	0.3662	40.8	6.04
12	55	168	393.84	0.6194	0.0055	0.0279	0.5860	94.6	1.1091	0.5137	0.0868	0.5086	45.8	6.60
14	60	150	332.93	0.5243	0.0039	0.0412	0.4792	91.4	0.9622	0.4440	0.1125	0.4057	42.2	7.29
16	55	143	309.71	0.4881	0.0045	0.0269	0.4567	93.6	0.9061	0.3950	0.1160	0.3951	43.6	7.82
Mean	59.0	155.3	352.82	0.5554	0.0057	0.0368	0.5129	92.31	1.0102	0.4718	0.1037	0.4346	42.98	6.89

Rats 1 and 2, 3 and 4, etc., are litter-mates.

* The figures in this column include 0.0046 g. Ca derived from the yeast extract.

† The figures in this column include 0.1588 g. P derived from the yeast extract.

RESULTS

The results of the experiment are given in Table I. Both groups grew at the same rate and consumed 5.8 mg. Ca and 10.5 mg. P per g. gain in weight.

It will be seen from the Table that the retentions of Ca were very similar in the two groups of rats, 91.95 % for the control group and 92.31 % for the vitamin C group. The difference of 0.36 % in favour of the vitamin C group has a standard error of the mean of ± 1.06 ["Student", 1908; 1925] and is obviously not significant. 44.25 % of the ingested P was retained by the control group of animals and 42.98 % by the vitamin C group. The difference of 1.27 % in favour of the control group has a standard error of ± 1.20 with odds of 1 : 3 against its having statistical significance.

It is quite clear that the addition of vitamin C in this experiment did not improve the retention of either Ca or P when Ca was fed at a sub-optimal level in the presence of adequate P.

These findings were confirmed by the results of the carcass analyses. Details of the analyses are not included here as, apart from their confirmatory value, they contribute little to the results.

The retentions of both Ca and P were slightly lower in the present work than those observed by us previously with the same diet [Henry & Kon, 1939]. This may have been due to the use of a different sample of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. The higher faecal losses of Ca and P point to the possibility that a larger proportion of the salt passed unchanged through the gut. In this experiment the average weight of faeces was definitely higher than in the previous one (7 g. against 5.5 g.). This would also contribute to a larger faecal loss of P [Henry & Kon, 1939].

SUMMARY

The addition of 2 mg. daily of ascorbic acid had no effect on the retention of Ca by rats receiving a diet low in Ca, adequate in P and devoid of vitamin C. The Ca was mainly derived from $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$.

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CCVII. VITAMINS IN RAT'S AND IN GUINEA-PIG'S MILK¹

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THE development of a technique for the satisfactory milking of small laboratory animals [Cox & Mueller, 1937; Temple & Kon, 1937] and the recent progress in the chemical and physical estimation of various vitamins have made it possible to measure several of these factors in the milk of rats and of guinea-pigs.

We have examined the milks of both species for vitamin A and carotene, vitamin B₁, riboflavin and vitamin C. We have also studied the effect of changes in the diet on the levels of these factors in rat's milk.

EXPERIMENTAL

(1) *The guinea-pig*

Guinea-pigs were generally milked about the 4th day of lactation, some being used several times at intervals. For milking in the morning the doe was separated from her young on the previous evening at 5 p.m. Their food consisted of a dry mash, the composition of which is given in Table I. In addition they were allowed unlimited hay and water and about 1 oz. of fresh cabbage daily.

Table I. *Composition of the guinea-pig diet*

Bran	27
Crushed oats	27
Lucerne meal	13
Linseed cake	13
Flaked maize	13
Fish meal	7

Approximately 1% cod liver oil was mixed in immediately before feeding.

Twenty milkings yielded 99.6 ml. of milk, i.e. an average of 5 ml. The best yield from one guinea-pig was 12 ml.

(a) *Vitamin A and carotene.* 15.5 ml. of milk were diluted with 2 vol. of water and the fat was extracted by the method of Gillam *et al.* [1938]. The yield was 1.27 g. or 8.2%. Our usual technique [Gillam *et al.* 1937] showed a content of 4 yellow and 42 blue Moore [1930] units per g. fat, equivalent to 0.143 mg. carotene and 1.08 mg. vitamin A per 100 g. of fat [Bartlett *et al.* 1938].

(b) *Vitamin B₁* was estimated fluorimetrically [Henry *et al.* 1938; Houston & Kon, 1939; Houston *et al.* 1939, 1], both the form which reacts in the Jansen [1936] technique ("free" vitamin B₁) and the total aneurin being measured. In February 1938 a figure of 18.4 I.U./100 ml. was obtained for the "free" form in the pooled milk of 2 guinea-pigs. In October of the same year the readings were 15.4 I.U./100 ml. as "free" aneurin and 22.4 I.U./100 ml. after incubation with pepsin and takaphosphatase (takadiastase of Parke Davis and Co.). These figures are not greatly different from those for cow's milk (Table VII).

¹ Read in part before the Biochemical Society, 11 March 1938 [Houston *et al.* 1938].

When the fluorimetric test was applied to the guinea-pig milk much fluorescent material was found in the aqueous layer after extraction with *isobutanol*. The fluorescence was not removed by incubation with pepsin or with *takaphosphatase* and it is therefore unlikely that more than a fraction of it was due to *öocarboxylase*. Similar blue fluorescent compounds are found in products of vegetable origin.

(c) *Riboflavin (lactoflavin)*. The method of Whitnah *et al.* [1937] was used but the readings were taken in the fluorimeter as used by Henry *et al.* [1939]. Estimations carried out in February 1938 showed a content of 86 µg./100 ml., a value lower than, but not greatly different from, that for cow's milk (Table VII).

(d) *Vitamin C*. Readings [Kon & Watson, 1936] on milk samples collected in the absence of light from 4 guinea-pigs in February 1938 gave values of 28–31 mg./100 ml., all in the reduced form. These values are very high, more than 10 times the normal for cow's milk (Table VII) and about half that of an average lemon juice. As the reducing substance in guinea-pig's milk is destroyed by light in the usual fashion it is unlikely that it is an artefact. Rasmussen *et al.* [1938] have since reported similar and even higher values for guinea-pig's milk. They found that the vitamin C content is influenced by the diet, and by feeding to a guinea-pig 40 mg. of ascorbic acid daily, beginning 17 days before parturition, brought the concentration in milk to 75 mg./100 ml.

(2) *The rat*

The bulk of the milk for the estimation of the normal vitamin levels was obtained from stock colony rats on the day following weaning, that is on the 23rd day of lactation. Where the effects of dietary changes were studied, milkings were started earlier, generally on the 13th day of lactation, and continued on alternate days. In all cases the doe was separated from the young for 16–18 hr. before milking. 129 milkings were carried out, yielding 173.1 ml. of milk, an average of 1.3 ml. per milking. The best yield was 6.5 ml. An analysis of the milk gave 13.8 % fat and 27.9 % total solids, in good agreement with the figures of Cox & Mueller [1937]. The diet of the stock colony has been described by Folley *et al.* [1938]. The methods of estimating the various vitamins were the same as for guinea-pig's milk.

(a) *Vitamin A and carotene*. From 19.1 ml. of milk obtained from 17 rats in January 1938, 2.23 g. of fat were extracted which contained 36 Moore blue units of vitamin A per g. or 0.92 mg. of vitamin A per 100 g. Calculated on milk this is rather higher than the value for cow's milk (Table VII). The fat was quite colourless and contained no measurable carotene, which indicates that the rat is, like the goat, more efficient than the cow in converting the pigment into vitamin A.

(b) *Vitamin B₁*. Results of tests are summarized in Table II.

Table II. *Estimation of vitamin B₁ in rat's milk*

Date	No. of rats	Day of lactation	Yield of milk ml.	Vitamin B ₁ (i.u./100 ml.)	
				"Free"	Total
27. i. 38	7	23rd	14.2	70.5	—
21. vi. 38	2	17th	3.0	44.8	—
21. vi. 38	2	17th	4.9	41.7	—
31. x. 38	3	23rd	6.1	55.3	77.8
8. v. 39	4	15th	6.0	33.3	53.1
9. v. 39	4	15th	6.0	45.3	49.1
			Mean	48.4	60.0

Only the "free" vitamin B₁ was at first estimated. Both it and the total vary fairly considerably but are in any case 3-4 times higher than in cow's milk (Table VII). The figures have a tendency to rise with progressing lactation.

The effects of diets deficient in vitamin B₁ and of feeding large quantities of the factor were also tested. The secretion of aneurin in milk during vitamin B₁ deficiency is shown in Table III.

Table III. *Vitamin B₁ content of milk secreted by rats receiving diets deficient in vitamin B₁*

Exp. no.	No. of rats	Date	Day of lactation	Days on deficient diet	Yield of milk ml.	Vitamin B ₁ (i.v./100 ml.)	
						"Free"	Total
1*	2	21. vi. 38	17th	0	4.9	41.7	—
		23. vi. 38	19th	2	1.0	20.6	—
		25. vi. 39	21st	4	2.7	22.9	—
2†	4	9. v. 39	14th	0	6.0	45.3	49.1
		11. v. 39	16th	2	1.9	27.3	32.1
		17. v. 39	22nd	8	6.0	Trace	Trace

* Other members of the vitamin B complex supplied as autoclaved yeast.

† Other members of the vitamin B complex supplied as autoclaved whey.

It is evident from these figures that the secretion of vitamin B₁ into the milk drops rapidly when little or none is present in the diet. This is in agreement with previous experience [cf. Hartwell, 1925].

The effect of feeding relatively large doses of crystalline aneurin to lactating rats receiving the stock diet is summarized in Table IV. The aneurin, dissolved in a few drops of water at pH 5, was fed by pipette.

Table IV. *The vitamin B₁ content of milk of rats receiving massive doses of aneurin*

Exp. no.	No. of rats	Date	Day of lactation	Days on experiment	Total additional vitamin B ₁ fed, i.v. per rat	Yield of milk ml.	Vitamin B ₁ in milk i.v./100 ml.	
							"Free"	Total
1	2	21. vi. 38	17th	0	0	3.0	44.8	—
		23. vi. 38	19th	2	333	2.0	40.6	—
		25. vi. 38	21st	4	666	1.8	39.1	—
2	5	8. v. 39	15th	0	0	6.0	33.3	53.1
		10. v. 39	17th	2	530	2.9	43.2	47.1
		12. v. 39	19th	4	1060	4.8	43.6	54.1
		16. v. 39	23rd	8	2120	4.9	40.2	47.1

It is surprising to find that the massive doses fed did not raise the vitamin B₁ level of the milk, especially as the effect of cutting down the supply was so definite. This may mean that the vitamin B₁ content of rat's milk is under physiological control and is not allowed to rise beyond the level already reached on the stock diet. The possibility of an effect similar to that reported by Perla & Sandberg [1939] is by no means excluded.

(c) *Riboflavin (lactoflavin)*. One assay in January 1938 on the pooled milk of 5 rats on the 23rd day of lactation gave a value of 407 $\mu\text{g.}/100$ ml. Another in June of the same year gave for the combined milk of 2 rats on the 17th day of lactation double this value, 807 $\mu\text{g.}/100$ ml. Data in Table V show that the feeding to stock rats of additional riboflavin definitely increases the level of this factor in milk and it is possible that the variation was due to fluctuations

in riboflavin content of the stock diet, especially of such a component as beef liver. To test the effect of increased intake, pure riboflavin dissolved in a small quantity of water was fed by pipette (Table V).

Table V. *Effect on the level in milk of feeding of additional riboflavin to lactating stock rats*

No. of rats	Date	Day of lactation	Days on experiment	Total additional riboflavin fed $\mu\text{g. per rat}$	Yield of milk ml.	Riboflavin in milk $\mu\text{g./100 ml.}$
2	21. vi. 38	17th	0	0	3.0	807
	23. vi. 38	19th	2	1000	2.5	1318
	25. vi. 38	21st	4	2000	2.0	1426

The feeding of riboflavin nearly doubled the already high content of the milk. Rat's milk is a richer source of this factor than cow's milk (Table VII) but we have found up to 800 $\mu\text{g./100 ml.}$ in the colostrum of Shorthorn cows [Houston *et al.* 1939, 2].

(d) *Vitamin C.* Rat's milk contains very little vitamin C. A sample of 8.4 ml. collected in January 1938 in the absence of light from 6 rats (23rd day of lactation) contained 0.25 mg./100 ml. of reduced and 0.39 mg./100 ml. of total ascorbic acid. The corresponding figures in June of the same year obtained on a 4 ml. sample from 2 rats on the 17th day of lactation amounted to 0.31 mg./100 ml. for both reduced and total ascorbic acid, showing that the reversibly oxidized form was not present. This is only about one-eighth of the amount found in cow's milk. The level is not increased by feeding liberal quantities of the crystalline substance dissolved in a few drops of water, as can be seen from Table VI.

Table VI. *Effect of feeding of ascorbic acid to lactating rats on the vitamin C content of milk*

No. of rats	Date	Day of lactation	Days on experiment	Total additional vitamin C fed mg. per rat	Yield of milk ml.	Vitamin C in milk mg./100 ml.	
						Reduced	Total
2	21. vi. 38	17th	0	0	4.0	0.31	0.31
	23. vi. 38	19th	2	50	1.5	0.32	0.33
	25. vi. 38	21st	4	100	0.5	—	<0.3

The last figure is very doubtful owing to the very small yield of milk on that day. There is no doubt, however, that the feeding of 100 mg. of ascorbic acid to each of 2 rats had no effect on the level of the substance in the milk. In this respect the finding of Longenecker *et al.* [1939] that rats excrete in the urine only a small quantity of vitamin C when fed as much as 100 mg. of the pure substance is of interest. It is generally agreed that the rat does not require preformed vitamin C in the diet and both the deficiency of the substance in the normal milk and the lack of response to the feeding of large quantities can be readily understood on teleological grounds. One might be tempted to suggest a similar explanation of the relatively enormous concentration of vitamin C in the milk of the guinea-pig, but one should remember that the young of this species begins to nibble solid food almost immediately after birth. Whatever the explanation, the striking fact remains that in guinea-pig's milk there is 70 times as much vitamin C as in the milk of the rat.

(3) *Comparison of rat's, guinea-pig's and cow's milk*

In Table VII we have compared the vitamin values found by us for rat's and guinea-pig's milks with those for cow's milk.

Table VII. *Comparison of the concentrations of certain vitamins in rat's, guinea-pig's and cow's milks*

Milk	Fat %	Vitamin A mg./100 ml.	Carotene mg./100 ml.	Vitamin B ₁ I.U./100 ml.	Riboflavin mg./100 ml.	Vitamin C mg./100 ml.
Rat	14.0	0.13	Not measurable	50-80	0.4-0.8	0.4
Guinea-pig	8.0	0.09	0.011	20	0.085	29.0
Cow (Shorthorn)	3.8	0.02-0.07	0.006-0.03	12-20	0.1-0.15	2.5

SUMMARY

1. The following values were obtained by physical and chemical methods for the concentration per 100 ml. of vitamin A, carotene, vitamin B₁, riboflavin and vitamin C respectively in rat's and guinea-pig's milks, viz. rat: 0.13 mg.; not measurable; 50-80 I.U.; 0.4-0.8 mg.; 0.4 mg.; guinea-pig: 0.09 mg.; 0.011 mg.; 20 I.U.; 0.085 mg.; 29.0 mg.

2. Compared with cow's milk rat's milk is richer in vitamin B₁ and riboflavin but contains much less vitamin C.

3. Guinea-pig's milk on the contrary contains relatively enormous quantities of vitamin C, about half the amount present in an average lemon juice.

4. Feeding large quantities of riboflavin to lactating rats definitely increased the concentration of this factor in milk but generous administration of vitamin C and of vitamin B₁ was without effect. On the other hand the vitamin B₁ content of rat's milk dropped sharply when a vitamin B₁-deficient diet was fed.

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CCVIII. PHYTIC ACID AND THE RICKETS-PRODUCING ACTION OF CEREALS

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THE fact that cereals produce rickets in young animals was demonstrated in 1920 when it was found that increasing the amount of these substances in diets which were deficient in the antirachitic vitamin led to an increase in the intensity of rickets [Mellanby, 1920]. At that time the explanation offered to account for the phenomenon was that cereals increased the rate of growth of animals, yet they could not at the same time supply the necessary elements to ensure the perfect formation of the bones. The greater the rate of growth induced by feeding more cereal, the greater was the demand for the food factors necessary for bone and tooth calcification and the more abnormal therefore became the condition of the bones and teeth. While this explanation undoubtedly accounted in part for the rachitogenic action of cereals in these experiments, it soon became clear that it could not explain all the facts, especially the observation that different cereals eaten in amounts which produced similar rates of growth produced widely different intensities of rickets. Experiment showed that cereals could be graded in their rickets-producing effect, oatmeal being the worst of those tested, white flour and rice having the least interfering effect on calcification, and whole-meal wheat flour being more rachitogenic than white flour [Mellanby, 1922; 1925].

It has long been known, of course, that the degree of calcification produced by a diet depends in part on the amounts and relative proportions of Ca and P in the diet. Examination of these graded effects of cereals on bone and tooth calcification, however, soon revealed that they could not be explained simply in terms of Ca and P contents or by the different ratios of these elements in cereals. On the whole, the cereals with the largest amounts of Ca and P, such as oatmeal, maize and wheat germ, caused the lowest Ca and P retention in the bones and teeth—an enigma which threw doubt on the nutritional significance of the values obtained by chemical analysis for the total Ca and P contents of foods.

In view of these facts it seemed clear that the rachitogenic action of cereals could not be fully explained by their growth-promoting action or mineral content, and it was concluded that some of the more strongly rachitogenic of the group, especially oatmeal and maize, must contain an active rickets-producing substance. This view of the presence of some positive, toxic, rickets-producing factor in cereals was strengthened by the observation that the effect could be destroyed by boiling the cereal for some time with dilute HCl [Mellanby, 1925]. On feeding the neutralized product (the NaCl content of the control diets being, of course, made up to the same value), it was found that the rachitogenic action of the cereal had been largely destroyed. Attempts made to isolate such a factor, and to demonstrate its action when separated from the cereal, at that time failed. In spite of this failure, some of the conditions which affected the activity of the rachitogenic substance in cereals were disclosed and these facts were useful in determining the further progress of the work.

In the first place it was found that the rickets-producing effect of cereals, even of those with the greatest activity, could be completely antagonized by the addition of sufficient vitamin D to the diet. In diets containing only a little vitamin D, the addition of one of the more powerfully rachitogenic cereals supplied a factor which counteracted the calcifying action of the vitamin and produced rickets. On adding more vitamin D to such diets, the effect of the vitamin became predominant and normal calcification resulted. The Ca and P of the cereals and other dietary constituents, which were lost to the body in the presence of the cereal factor when vitamin D was deficient, were absorbed and became incorporated in bones and teeth when the vitamin was present in sufficient quantity. It was this property of antagonizing the action of vitamin D which suggested the name anticalcifying toxamin for the unknown substance in cereals, for it was clearly a toxic substance, in that it interfered with Ca and P metabolism and thereby made animals abnormal, and yet its harmful action could be counteracted by a particular vitamin.

A second point of interest is that the cereal effect can be largely antagonized by increasing the Ca of the diet by adding calcium carbonate or phosphate. With a cereal diet, if vitamin D is absent or very deficient, perfect teeth and bones are not produced even in the presence of abundance of Ca, but with the deficiency of vitamin D the extra Ca greatly improves the calcification processes [Mellanby, 1925].

It was mentioned above that boiling cereals with acid destroys their rachitogenic activity. Another observation relating to the destruction of the anticalcifying activity may be mentioned. It is that malted cereals have usually lost their rickets-producing effect. A closer examination of this revealed that germination of grains such as oats does not in itself bring about the loss of activity, but if the germinated oats are crushed and allowed to stand for 2 days at room temperature, the rachitogenic action disappears [Mellanby, 1929]. Templin & Steenbock [1933] later found that with maize, similarly, germination did not destroy the rachitogenic action but that the activity was lost on subsequent autolysis. They showed that this disappearance of rachitogenic action was accompanied by a change of organic P in the maize to the inorganic form.

Steenbock *et al.* [1930] had previously drawn attention to the possibility that inorganic phosphate added to the diet may not be equivalent in physiological properties to the organic phosphoric compounds in cereals. There was indeed already some evidence on this point.

It has long been known that a large part of the organic P of cereals, and of seeds in general, occurs in the form of phytic acid (inositolhexaphosphoric acid). This acid appears to be present, at any rate in part, as the CaMg salt which is known as phytin.

The work of Starkenstein [1910] and of Plimmer [1913] had provided evidence that this substance, phytin, when fed to animals, is not directly absorbed from the alimentary canal, and that such breakdown of the compound as occurs in the intestine (leading to the formation of inositol and phosphoric acid) is probably largely the result of bacterial action.

Bruce & Callow [1934] followed up the deduction from the work of Steenbock *et al.* [1930] that cereals contain a form of P less available than inorganic phosphate. A high proportion of some such compound containing non-available P in a given cereal would mean that this cereal would probably show little calcifying power when added to a basal diet low in P, and under these experimental conditions it would be classed as a relatively rachitogenic cereal. Bruce & Callow tested this question, having particular regard to the possibility that the non-

available cereal P might be phytic acid P. They used rats as experimental animals and fed them on a high Ca-low P diet which was deficient in vitamin D. Such a diet by itself produces rickets in rats, the intensity of rickets being diminished by the addition of inorganic phosphate in sufficient amounts to bring the Ca:P ratio within more normal limits. They found that on adding different P-containing compounds to the diet there was a reduction in the intensity of the disease in proportion to the availability of the P of the compound, and that phytin (CaMg inositolhexaphosphate) or sodium phytate caused only a small decrease in the rachitic potency of the diet, while addition of disodium hydrogen phosphate having the same content of P caused a large reduction in rachitic action. Their results established the fact that, under these dietetic conditions, the P of phytin was much less available to the animal than that of sodium phosphate and that the relative effect of these substances in combating rickets of a high Ca-low P type was dependent on this difference of availability. This comparative unavailability of phytic acid P holds also for man, as was shown shortly afterwards by McCance & Widdowson [1935], who determined the actual amount of phytic acid P in the faeces after adding phytin to the diet of human beings. They found that 20–60 % of the phytin was excreted unchanged in the faeces and suggested that much of the remaining phytin P may also have been unabsorbed and have been present in the faeces in other forms.

The question of unavailability to animals of phytin P in the diet was also investigated by Lowe & Steenbock [1936, 1] who found that phytin P is partially available to the rat on a low Ca ration, but that the addition of CaCO_3 makes it almost completely unavailable.

All these experimental results made it clear that the statement of Steenbock *et al.* [1930] that the physiological properties of the compounds in cereals containing P were probably different from those of inorganic phosphates was certainly true.

Further, however, the fact that phytic acid was converted into inorganic phosphoric acid and inositol when boiled with dilute HCl or by the action of phytase fitted in well with the earlier observations that the rachitogenic factor of cereals is destroyed by boiling with HCl or by exposure to the action of the cereal enzymes after germination, and the results seemed to indicate at first sight that the rachitogenic factor of cereals was in fact phytin.

On further consideration it became clear, however, that these various experiments on the relative availability of P in different compounds had little direct bearing on the positive, rickets-producing action of cereals described by Mellanby. Quite apart from the phytin P, there was abundant P in other forms to produce good bones in Mellanby's experiments on dogs; moreover, unlike Bruce & Callow, and Steenbock, Mellanby found that, under his experimental conditions, the addition of inorganic phosphate produced no improvement in calcification. The high Ca-low P diets used by Bruce & Callow to produce rickets in rats are not the types of diet which usually produce rickets in children, and rickets in children cannot normally be improved, much less cured, by adding inorganic phosphate to the diet. From the point of view of its relation to human nutrition, the high Ca-low P diet used for studying rickets in rats is a very artificial one. The dietary conditions in Mellanby's experiments with puppies approach much more closely the conditions leading to rickets in children.

The point at issue is clearly not whether phytin P is available but whether phytin itself in cereals is responsible for their positive, rickets-producing effect under ordinary dietetic conditions. Bruce & Callow themselves pointed out that their interpretation of the phytin effect as being due to its unavailable P was

based on results obtained with the high Ca-low P diets generally used in studying rickets in rats. They attempted to extend their observations to low Ca-high P diets, but the results obtained were not definite. They suggested, however, that under such conditions the action of phytic acid might be different, and drew attention to the work of Starkenstein [1914] on the possible action of phytic acid in precipitating Ca or rendering it un-ionized.

Such a possibility seemed much more in accordance with the observations of Mellanby, particularly with the fact that adding Ca to the diet counteracts the rachitogenic effect of the cereal. Indeed, with this possibility in mind, we carried out experiments in which we tried to isolate the rickets-producing factor of HCl-extracts of oatmeal by precipitation of the neutralized, filtered extract with CaCl_2 . On feeding the product after removal of Ca as oxalate, we at that time got no definite result, however, probably because the substance was given in insufficient amounts.

An observation which encouraged the view that phytic acid was in some way concerned with the cereal action was the fact that Holst [1927] had shown that the active factor could be extracted from oatmeal by cold dilute HCl, for earlier workers had used this very method for extracting phytic acid from foodstuffs prior to its estimation by iron titration [Heubner & Stadler, 1914].

It seemed desirable, therefore, to try the effect of feeding phytic acid and phytin to dogs using the diets with the more natural Ca:P ratios. If then phytic acid showed a rachitogenic action and this action were due to interference with Ca absorption by precipitation of the base, it would be expected in such experiments, unlike those with a high Ca-low P ratio, that phytin, the CaMg salt, would on the other hand show little or no rachitogenic action.

It will be seen in Exp. I that such results were actually obtained.

EXPERIMENTAL METHODS

In the experiments described in this paper, carried out to test the effect on the development of rickets of the addition to the diet of phytic acid compounds and fractions prepared from cereals, puppies were used, and the technique employed was essentially the same as that previously described by one of us [Mellanby, 1921; 1925]. Differences in susceptibility to rickets between different litters of animals demand that each experiment be done on dogs from a single litter, in order to justify comparison between the final conditions of the control animal or animals and those whose diet contained the various added substances. There are differences in susceptibility to rickets, even among individual members of a litter, so that repetition and confirmation of each result are essential. Where an observation has not been confirmed on a number of occasions, this fact has been stated in the text. This difference in susceptibility and, to a less extent, difference in form of manifestation of the disease, also necessitate that appraisal of the degree of rickets produced, and of the divergence from the normal, should only be made after various forms of examination. These were (1) the appearance of the dog, including the degree of leg deformity and the size of the epiphyseal ends of the bones and the costo-chondral junctions, (2) the radiographic appearance of the epiphyseal ends of the bones, (3) the degree of calcification of bones and (4) the histological appearance of the bones. In the results recorded below only the radiographic appearances of the bones at death and the results of estimations of their Ca contents are given. In general these findings were substantiated by the appearance of the animals and the histological examination of the bones.

As regards the degree of calcification of the bones, this is recorded as the ratio A/R, the ratio of the weight of ash of the bone to the weight of dry fat-extracted bone minus ash.

It has been previously pointed out that rickets in young animals is a disease associated with growth, so that it is essential in such work, where comparisons have to be made, to adjust the amount of food given so that each animal eats the same quantity of the basal diet, and that the growth (as measured by weight increase) of the animals in a litter is as similar as possible. It is usually not difficult to attain this in experiments of the duration of those in the present enquiry. Growth curves have not been given in this publication but the similarity in rates of growth can be seen in the figures of weight increase recorded. Usually there is little or no discrepancy between the final weight and the maximum weight of each animal but, where there has been loss of weight before the completion of an experiment, this fact is recorded in the experimental results.

There has been one special difficulty about the present experiments which needs to be mentioned. In order to test the effect of some substance in increasing the intensity of rickets it is necessary to make the control diets of such a nature that rickets in these animals is either produced only slightly or just prevented by a narrow margin. If, for instance, the control animals get severe rickets, the effect of adding a rickets-producing substance to the diet of other animals of the litter may be lost because all animals may be so severely affected. If, on the other hand, the basal diet is too antirachitic, the effect of the substance to be tested may be so far overcome as to escape detection. For this reason the amounts or proportions of separated milk powder and cabbage in the diets were sometimes altered. This change affected, of course, every member of the litter. Experience in choosing diets compatible with the production of varying degrees of rickets in puppies has, generally speaking, allowed this difficulty to be avoided in the present work.

Preparation of sodium phytate from commercial phytin

The phytin used in Exps. 1-3 was a commercial preparation (Phytin "Ciba"). The sodium phytate for these three experiments was made by suspending 75 g. of the same phytin in 750 ml. water, stirring with *N* HCl till dissolved and adding *N* NaOH gradually with stirring till a permanent precipitate just formed. A warm solution of sodium oxalate equivalent to the Ca in the phytin was added, and then *N* NaOH to bring the solution to about pH 5. The solution was allowed to stand and was filtered, the precipitate being washed with water. The combined filtrate and washings were neutralized, allowed to stand overnight and filtered again. The solution was then diluted so that 20 ml. were equivalent to 0.6 g. of the original phytin, and was stored in the refrigerator.

In the first three experiments the sodium phytate and phytin were fed in amounts based on the assumption that oatmeal contained 0.6% phytin. Soon afterwards the method of McCance & Widdowson [1935] for phytin estimation appeared and, on using this to determine the phytate P of our oatmeal, we found that we had been feeding the sodium phytate and the phytin at only about half the oatmeal equivalent of phytate P. But for this, the rachitogenic effect of sodium phytate would no doubt have been even more marked. In all experiments after Exp. 3, we estimated the phytic P in each phytate preparation and in each batch of oatmeal.

Exp. 1. Effects on calcification of phytin and sodium phytate

The basal diet consisted of separated milk powder 15-26 g., white flour 100-150 g., lean meat 15-22.5 g., orange juice 6 ml., yeast 5-7.5 g., NaCl 2 g.,

peanut oil 10 ml., cabbage 20 g. The dog receiving calcium lactate was given 0.554 g. increasing up to 0.831 g. as the amount of cereal increased. (The changes in amount represent the increases with advancing age of the puppies.) Age at beginning of experiment, 8 weeks. Duration of experiment, 13 weeks 4 days.

Results of Exp. 1

No. of puppy	Addition to basal diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray at death
2121	Sodium phytate (as in No. 2124) plus calcium lactate (0.554 %)	2600	7100	7200	1.39	Slight rickets
2122	None	2520	7340	7340	1.08	Moderate rickets
2123	Phytin 0.6-0.9 g. daily	2800	7820	7820	1.33	Slight rickets
2124	Sodium phytate equivalent to 0.6-0.9 g. phytin daily	2740	7500	7500	0.76	Bad rickets

It will be seen that phytin added daily to the diet to the amount of 0.6 g. increasing to 0.9 g. has had an antirachitic influence and has actually improved calcification. On the other hand, the addition of sodium phytate equivalent to 0.6-0.9 g. phytin has increased the rickets of 2124 and greatly diminished the calcium in the bones. The addition of Ca in the diet of 2121 has abolished the rachitogenic action of sodium phytate.

Exp. 2. Effects on calcification of phytin and sodium phytate

The basal diet in this series was separated milk powder 12.5-20 g., white flour 60-100 g., lean meat 10-15 g., baker's yeast 3-5 g., NaCl 1-2 g., orange juice 6 ml., peanut oil 10 ml., cabbage 14-20 g. Age of puppies at beginning of experiment, 9 weeks; duration of experiment, 20 weeks.

Results of Exp. 2

No. of puppy	Addition to basal diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray at death
2125	None	1840	5080	5100	1.52	Normal
2126	Phytin 0.36-0.6 g. daily	1660	4560	4560	1.54	Normal
2127	Sodium phytate equivalent in amount to 0.36-0.6 g. phytin daily	1560	5520	5520	1.30	Slight rickets
2128	Oatmeal replaced white flour	2320	5360	5380	0.88	Bad rickets

In this experiment it will be seen that (1) phytin has not increased the rickets, (2) sodium phytate has had a rachitogenic action but not to the extent of the diet in which white flour was replaced by oatmeal.

Exp. 3. Effects on calcification of (a) phytin, (b) sodium phytate, and (c) sodium phytate after boiling in 2% HCl

Basal diet: separated milk powder 21-26 g., bread 160-200 g., lean meat 18-22.5 g., baker's yeast 6-7.5 g., cabbage 28-21 g., peanut oil 10 ml., NaCl 1-2 g., orange juice 6 ml. Age of puppies at beginning of experiment, 10 weeks; duration of experiment, 8½ weeks.

Results of Exp. 3

No. of puppy	Addition to basal diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray at death
2143	None	3020	4100	4600	1.10	Normal
2144	Phytin 0.72-0.9 g. daily	3400	5060	5400	1.20	Normal
2145	Sodium phytate equivalent to 0.72-0.9 g. phytin	4120	5740	5800	1.02	Definite rickets
2146	Sodium phytate boiled $\frac{1}{2}$ hr. with 2% HCl equivalent to phytin 0.72-0.9 g.	4120	5900	5900	0.92	Rickets slightly worse than 2145

In this experiment also it is seen that phytin has not had a rickets-producing effect. Sodium phytate on the other hand has interfered with calcification. In 2146 the sodium phytate was boiled with 2% HCl for $\frac{1}{2}$ hr. in order to see whether the rickets-producing effect of sodium phytate would be destroyed, for earlier experiments had shown that the rachitogenic effect of oatmeal is reduced by boiling with acid. This experiment indicates that such treatment has not destroyed the rachitogenic action of sodium phytate. Subsequent experiments showed that only a small proportion of the sodium phytate is hydrolysed by boiling for $\frac{1}{2}$ hr. with 2% HCl.

*Preparation of purified alkaline sodium phytate and of phytic acid
from commercial phytin*

The sodium phytate used in the next experiment and in Exps. 5 and 6 was prepared from commercial phytin by precipitation of the iron salt from an acid solution, and was considerably purer than that used in the preceding experiments. The method was based on that described by Posternak [1921]. 200 g. of phytin were stirred into 2 l. of *N*/3 HCl and a small excess of ferric chloride (2 l. of 7% FeCl₃ solution) was stirred in. The precipitate of iron phytate was filtered on a large Büchner funnel, suspended by mechanical stirring in 3 l. of *N*/6 HCl, filtered and thoroughly suspended in 2 l. water. An excess of 40% NaOH (about 350 ml.) was added gradually and the mixture was stirred for $\frac{1}{2}$ -1 hr.; sufficient 5*N* HCl (about 50 ml.) was then added to make the reaction just alkaline to thymolphthalein (about pH 9). 100 g. NaCl were added (to assist in the precipitation of the colloidal ferric hydroxide) and the mixture was filtered by suction. The filtrate was usually practically free from iron, though an occasional preparation required further treatment with acid or alkali or by warming in order to precipitate all the colloidal iron. The precipitate was washed by stirring with 1 l. of water containing 10 ml. of 40% NaOH and then brought back to about pH 9 and filtered, and the combined filtrate and washings were treated with half their volume of absolute alcohol and allowed to stand overnight in the refrigerator. The alcohol was poured off from the semi-crystalline alkaline sodium phytate, which was then washed with a little 33% (by vol.) alcohol, dissolved in 100 ml. hot distilled water, warmed in a dish on the water bath to drive off residual alcohol and made up to about 300 ml. with water. A small volume of the syrupy solution was measured out in a blood pipette for determination of phytin P by the method of McCance & Widdowson [1935]. The solution usually contained about 45-50% anhydrous sodium phytate, and was practically free from inorganic P. It was diluted before mixing each day with the previously cooked basal diet and was fed in quantities equivalent to the phytic P eaten by the oatmeal control dog.

A portion of the solution was converted into free phytic acid by stirring in conc. HCl till the solution was just acid to Töpfer's indicator. The neutral Na phytate and phytic acid used in Exps. 4-7 contained NaCl. In all these experiments therefore the salt content of the diet of each dog was made equal by the addition of NaCl where required.

Exp. 4. Effects on calcification of (a) phytic acid, (b) sodium phytate (alkaline)

In this experiment the sodium phytate prepared as just described was fed to dogs 2394 and 2397 as the alkaline salt in which all the 12 hydrogen atoms of the phosphoric acid groups were replaced by sodium. Free phytic acid was fed to dog No. 2396. Basal diet: separated milk powder 16-27.5 g., polished rice 68-120 g., lean meat 13-23 g., cabbage 18-32 g., yeast 4-10 g., orange juice 6 ml., NaCl 1-2 g., peanut oil 10 ml. Age of puppies at beginning of experiment, 10 weeks; duration of feeding, 18 weeks. The oatmeal contained 0.224 % phytic P and the sodium phytate and phytic acid were fed in amounts equivalent as regards phytic P.

Results of Exp. 4

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2393	Oatmeal replaced rice	2210	7220	7220	1.15	Slight rickets
2394	Alkaline sodium phytate equivalent to that in oatmeal of 2393	1900	4830	5180	1.40	Normal
2395	No addition	2100	6120	6140	1.50	Normal
2396	Phytic acid equivalent to that in oatmeal of 2393	2370	5140	5420	1.19	Slight rickets
2397	Alkaline sodium phytate as 2394	2460	6320	6560	1.36	Normal

The results show that (1) oatmeal has produced worse calcification than polished rice, (2) phytic acid has interfered with calcification, (3) alkaline sodium phytate (unlike the neutral sodium phytate of previous experiments) has not shown any rachitogenic effect.

More experiments, however, would be necessary before this absence of rickets-producing effect with alkaline sodium phytate could be accepted. In order to test the possibility that alkali itself could neutralize the rachitogenic effect of oatmeal, an experiment was done in which the oatmeal of the diet after cooking was mixed with NaOH to bring it to a pH of 9.5. Two puppies were given ordinary oatmeal in the diet and three were given alkaline oatmeal. No difference in the degrees of rickets in the puppies was observed after 8 weeks of feeding. Thus no evidence was obtained that the ordinary acidity of oatmeal was responsible in itself for the rachitogenic action of this food and that simply adding enough base to combine with any free phosphoric groups could antagonize the oatmeal action on calcification.

Exp. 5. The effects on calcification of (a) phytic acid, (b) sodium phytate (neutral)

In contrast to the previous experiment, the purified sodium phytate was added at a neutral reaction, i.e. with some only of its phosphoric acid groups combined with sodium.

Alkaline sodium phytate was first prepared from commercial phytin by means of iron phytate as in the previous experiment. The neutral sodium phytate was made by adding HCl to the alkaline salt till neutral to phenol red. The phytic acid was prepared in the same way as in the previous experiment.

Basal diet: separated milk powder 20 g., white flour 50–160 g., lean meat 20–10 g., cabbage 20–5 g., baker's yeast 2·5–8 g., peanut oil 10 ml., orange juice 6 ml. Age of puppies at beginning of experiment, 8 weeks; duration of experiment, 14 weeks.

The phytic P content of the diets of the animals receiving sodium phytate or phytic acid was equal to that of the control dog receiving oatmeal. The oatmeal contained 0·225 % phytic P.

Results of Exp. 5

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2448	Sodium phytate (neutral)	1540	4420	4480	1·12	Fairly bad rickets
2449	No addition	1800	5020	5020	1·27	Slight rickets
2450	Phytic acid	1780	5080	5080	1·10	Fairly bad rickets
2451	Phytic acid as 2450	1880	4920	4920	1·17	Fairly bad rickets
2452	Oatmeal replaced white flour	1900	6240	6240	0·79	Bad rickets
2453	Sodium phytate (neutral) as 2448	1960	4760	4760	1·19	Fairly bad rickets

Both sodium phytate (neutral) and phytic acid had rickets-producing effects of about the same degree of intensity, but they were not so powerful in this respect as oatmeal, although the amount of phytic acid or phytate added was equivalent to the amount present in the oatmeal eaten by 2452. The much greater growth of the oatmeal dog may be responsible for this discrepancy.

Exp. 6. Effects on calcification of (a) sodium phytate (neutral), and (b) phytic acid

This experiment was a repetition of the previous one; the experimental conditions were on the whole similar and the method of preparation of sodium phytate and phytic acid was the same. The same sample of oatmeal was used in the two experiments.

Basal diet: separated milk powder 20–25 g., white flour 110–150 g., lean meat 15–20 g., cabbage 10–20 g., baker's yeast 5–7·5 g., orange juice 6 ml., peanut oil 15 ml. Age of puppies at beginning of experiment, 10 weeks; duration of experiment, 7½ weeks. (Oatmeal, 0·225 % phytic P.)

Results of Exp. 6

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2515	Oatmeal replaced white flour	2140	5320	5320	0·84	Very bad rickets
2517	No addition	2400	5180	5180	0·97	Fairly bad rickets
2518	Sodium phytate (neutral)	2520	4740	4860	0·86	Fairly bad rickets, rather worse than 2517 but not so severe as 2515 or 2519
2519	Phytic acid	2480	5020	5020	0·85	Very bad rickets, almost as bad as 2515

In this short experiment both phytic acid and sodium phytate have had rickets-producing effects but the phytic acid effect was greater. The shortness of the experiment was due to the rapidity with which even the control animal 2517 developed rickets.

Having established from the foregoing experiments that the rachitogenic action of oatmeal in the diet could be largely imitated by feeding phytic acid or neutral sodium phytate, it seemed reasonable to attempt an isolation of the rickets-producing factor by working on the assumption that the cereal factor might be phytic acid or some chemically similar compound. This assumption seemed a likely one in view of the fact that cereals are known to be rich in phytic P.

The first step in such an attempt was to get the oatmeal phytate into solution. Extraction with dilute HCl seemed the obvious choice, for there was already evidence that the rachitogenic factor could be extracted from oatmeal with HCl [Holst, 1927; de Bruin & Bouman, 1937], but it was at first found impossible to obtain an extract which was filterable unless so large a volume of HCl were used relative to the amount of oatmeal that the preparation became too bulky to handle on the large scale necessary for feeding dogs. The difficulty was finally overcome by first treating the oatmeal with diastase to break down most of the starch, and then extracting with HCl. In this way it became possible to obtain clear HCl filtrates using reasonably small volumes of fluid.

Method of preparation of sodium phytate from oatmeal

In order to facilitate extraction with HCl and subsequent filtration, the oatmeal was first fat-extracted. This fat extraction was carried out industrially on large batches of finely ground oatmeal, sufficient unextracted oatmeal from the same sample being retained for feeding to the control dogs.

The defatted oatmeal was treated in batches of 3 kg. and was suspended in 4.5 l. of 0.1 % NaCl which contained 2 g. of a strong diastase preparation and which had previously been warmed to about 40°. (The diastase was a purified takadiastase preparation which was kindly given to us by Messrs Parke Davis and Company. It was about ten times as active as an equal weight of commercial takadiastase tablets.) The mixture was divided into five roughly equal parts and put into five large jars with lids. After adding about 3 ml. toluene to each jar and well mixing the contents, the jars were covered and incubated at 37° with occasional stirring. After 24 hr., when the mixture had become much more fluid, 60 ml. of conc. HCl were gradually added to each jar with vigorous stirring. After shaking mechanically for 2½ hr., the contents were filtered on Büchner funnels, filtration taking about 4 hr., and the filtrate was immediately neutralized to phenol red with 40 % NaOH. In order to keep the volume of filtrate as low as possible and to avoid prolonged contact with the acid, the residue was not washed, but the filtrate was measured and its oatmeal equivalent calculated, an amount of extract equivalent to nearly 60 % of the oatmeal usually being obtained. After storing overnight in a refrigerator, the cloudy solution was brought to pH 5 and an equal volume of N/6 HCl was then added. A small excess of 3 % FeCl₃ dissolved in N HCl was stirred in, the volume of FeCl₃ being 1/10 that of the diluted filtrate, making the final concentration of HCl in solution approximately N/6. After heating in a boiling water bath for 20 min. to flocculate the precipitate, the mixture was cooled and the iron precipitate was filtered off by suction, washed by resuspending in 2 l. N/6 HCl and filtered again.

To convert the ferric compound (which was contaminated with a considerable amount of protein) into the sodium salt, the moist precipitate in a batch equivalent to 3 kg. oatmeal (i.e. from the filtrate obtained from about 5 kg. oatmeal) was suspended thoroughly in 1 l. of distilled water, using an efficient mechanical stirrer, after which an excess of 40 % NaOH (about 80 ml.) was gradually stirred in. After stirring for ½ hr. the solution was brought to approximately pH 11.5

(orange red to alizarin yellow G, used as external indicator) with 5*N* HCl and was filtered by suction. The precipitate of ferric hydroxide was washed by suspending in 250 ml. water containing 5 ml. 40 % NaOH and filtered. The light brown filtrate and washings were brought back to pH 11.5 and the alkaline sodium compound was thrown out of solution by stirring in half a volume of absolute alcohol. After standing for 2 days, the alcohol layer was carefully poured off and the syrupy semi-crystalline residue was dissolved in a little warm water, neutralized with conc. HCl and warmed in a dish on the water bath for a short time to drive off residual alcohol. A sample was removed and analysed for phytic P. The final yield based on the phytic P content of the original fat-extracted oatmeal was only about 30–35 %, due partly of course to the fact that a large amount of the phytic P was rejected in the moist oatmeal residue and also to the fact that the extraction of phytate from oatmeal by HCl is apparently incomplete under the conditions used.

When required for feeding, the solution was diluted and mixed with the food in quantities equivalent in phytic P to 50 % of the oatmeal eaten by the control dog. Owing to the large losses during extraction and purification of sodium phytate from oatmeal, and owing to the large amount required for feeding dogs, it was not possible in most of the experiments using sodium phytate from oatmeal to give it in quantities equivalent to the phytate in the oatmeal eaten by the control animal. In most experiments, therefore, only half the oatmeal equivalent of sodium phytate or phytic acid was given.

Exp. 7. Effect on calcification of sodium phytate (prepared from oatmeal)

Basal diet: separated milk powder 20 g., white flour 77–161 g., lean meat 12–25 g., cabbage 17–35 g., orange juice 6 ml., peanut oil 9–15 ml., baker's yeast 3.6–7.5 g. Age of puppies at beginning of experiment, 9 weeks; duration of feeding, 16 weeks. (Oatmeal contained 0.306 % phytic P.)

Results of Exp. 7

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2570	No addition	1980	5540	5540	1.38	Moderate rickets
2571	Oatmeal replaced white flour	2400	5240	5520	0.92	Very severe rickets
2572	No addition	2390	6140	6140	1.49	Slight rickets rather less than 2570
2573	Sodium phytate (from oatmeal)	2390	6080	6080	1.17	Fairly severe rickets, not so bad as 2571 but worse than 2570 and 2572
2574	Sodium phytate as in 2573	2580	6580	6580	1.01	Healing rickets

It can be seen that the sodium phytate fraction made from oatmeal has interfered with bone calcification when added to the white flour basal diet and that the degree of rickets and deficient calcification of bone produced by the addition of sodium phytate equivalent to 50 % of the oatmeal eaten by 2571 are not so bad as in this oatmeal control dog.

Exp. 8. Effect on calcification of sodium phytate (neutral)

This experiment was carried out to compare the rachitogenic action of neutral sodium phytate made from commercial phytin when purified and fed under similar conditions to those under which sodium phytate from oatmeal was used in the preceding experiment. The sodium phytate was fed in the same amount, i.e. at a 50 % oatmeal level.

Commercial phytin was worked up in batches of 400 g. and was precipitated with FeCl_3 from $N/6$ HCl solution (total volume 3 l.), the precipitate then being thoroughly suspended in water, decomposed with NaOH , and the sodium phytate then being thrown out of solution by adding half a volume of alcohol. The details were essentially the same as in the corresponding stages in the preparation of sodium phytate from oatmeal described in Exp. 7, except that the ferric phytate was washed with water after washing with HCl and that the alkaline sodium phytate was neutralized before being thrown out of solution with alcohol. The latter change in technique gave a final product practically free from NaCl and avoided the need for adding extra NaCl to the other diets. The neutral sodium phytate was obtained as an almost colourless syrup and, unlike the alkaline salt, showed no tendency to crystallize. A preparation containing approximately 50 g. of phytate P was obtained from 400 g. of commercial phytin.

The sodium phytate was fed in an amount equivalent to 50 % of the usual phytate contained in a weight of oatmeal equal to the weight of white flour eaten by the dogs on the basal diet.

Basal diet was made up as follows: white flour 65 %, lean meat 10 %, baker's yeast 3 %, NaCl 1 %, cabbage 14 %, peanut oil 7.5 %. Increasing quantities of this mixture, i.e. from 80 to 260 g., were given as the animals grew. In addition, from the beginning to the end of the experiment each puppy received 6 ml. orange juice and 20 g. separated milk powder. Age of puppies at beginning of experiment, 8½ weeks; duration of experiment, 14 weeks. (Oatmeal contained 0.290 % phytic P.)

Results of Exp. 8

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2637	No addition	2000	6440	6440	1.19	Very slight rickets
2638	No addition	1820	6480	6480	1.13	Very slight rickets
2639	Sodium phytate (from phytin)	1980	6280	6280	1.07	Fairly severe rickets
2640	Sodium phytate as 2639 (from phytin)	2120	6560	6560	1.09	Fairly severe rickets

The depression in calcification in the bones produced by sodium phytate is not great in this experiment as judged by the A/R ratio. On the other hand, the radiographs (see Figs. 1-4) show clearly the increased amount of rickets produced by the addition of sodium phytate to the basal diet. This was similar to the effect produced by the sodium phytate fraction from oatmeal itself (see Exp. 7).

Exp. 9. Effect of purified sodium phytate (from oatmeal) on calcification

In this experiment, the sodium phytate (neutral) given to dogs 2644 and 2646 was prepared from oatmeal but was subjected to a much more rigid process of purification.

Preparation and properties of purified sodium phytate from oatmeal

The first stages in the preparation, involving digestion of defatted oatmeal with diastase, extraction of phytate with HCl and precipitation as ferric phytate, were carried out exactly as described in Exp. 7. The ferric phytate was treated in batches obtained from extracts equivalent to 6 kg. oatmeal, i.e. from filtrate obtained from about 10 kg. original defatted oatmeal. The precipitate (about 600 g. wet wt.) was thoroughly suspended in 2 l. tap water, a small excess of 40 % NaOH (about 130 ml.) was gradually stirred in, and, after continuing the stirring for 1-1½ hr., the solution was filtered on a large Büchner funnel. The ferric hydroxide was washed by stirring with 500 ml. water containing 10 ml. 40 % NaOH, filtered, and the combined filtrates were brought to about pH 11.5 (orange-red to alizarin yellow G) with conc. HCl. Half a volume of absolute alcohol was added with shaking and the mixture was allowed to stand for 2-3 days in the refrigerator, by which time the alkaline sodium phytate on the bottom of the vessel had become semi-crystalline. The alcohol layer was carefully poured off and the residue was dissolved in 400 ml. warm water and a moderate excess of *M* BaCl₂ (370 ml.) was stirred in. The precipitate was filtered, washed with a little 30 % (by vol.) alcohol and dissolved in the minimum volume of 3 % HCl (600-700 ml.), and the solution was filtered to remove traces of insoluble material. Barium phytate was then precipitated from the acid solution by stirring in an equal volume of absolute alcohol [Anderson, 1914]. The precipitate was filtered by suction, washed with 50 % alcohol and sucked as dry as possible, after which it was well suspended in 400 ml. warm distilled water and the barium completely removed by treatment with slightly more than the equivalent amount of 20*N* H₂SO₄ (about 30 ml.). After stirring for ½ hr., the BaSO₄ was centrifuged off, resuspended in water containing a few drops of H₂SO₄, again centrifuged, and the combined clear solution and washings were neutralized to phenol red with 40 % NaOH. The neutral sodium phytate was thrown out of solution by stirring in half a volume of alcohol, forming a heavy, clear, colourless, syrupy liquid, which usually contained about 150 mg. phytic P per ml. and contained no appreciable amount of inorganic or other non-phytic P.

The preparation was stored at 0° and diluted as required for feeding. The yield over the whole process, based on the phytate P content of the original defatted oatmeal, was about 35 %.

The neutral mixture of sodium salts did not show any tendency to crystallize, but from it the alkaline sodium phytate could readily be obtained crystalline by diluting a portion of the syrup with an equal volume of water, adding sufficient 40 % NaOH to bring the pH to about 11.5 and evaporating the solution over CaCl₂ in a vacuum desiccator. When crystallization had begun, the solution was warmed to redissolve the crystals and the dish was allowed to stand in the open in a warm room with occasional stirring. After several days the syrupy mass of fine crystals was filtered on a coarse sintered glass funnel, the product was cooled to 0°, stirred up with a few ml. of ice-cold water, quickly filtered under suction on a cold funnel and dried on a porous tile. The salt has a very high temperature coefficient of solubility and the loss on washing is great unless the temperature is kept low. For analysis, the salt was recrystallized by dissolving 6 g. in 3 ml. of warm water, allowing it to stand in the open for 2 days with occasional stirring and again filtering and washing in the cold. The air-dried salt gave the following results on analysis:

Loss of water after drying over H₂SO₄ and then at 120° = 38.42 %.

Ash after ignition = 50.69 %.

The hydrated salt melted at 56-59°, the melting point not being sharp.

According to Posternak [1921], the air-dried alkaline sodium phytate has the composition $C_8H_6O_{24}P_6Na_{12} \cdot 3H_2O + 35H_2O$, and melts at 58–59°.

Calculated loss for $35H_2O = 39.18\%$.

Calculated ash ($3Na_4P_2O_7$) = 49.63 %.

On titration with $N/10$ HCl to a faint rose colour with methyl orange, 5.96 equivalents of acid were required. (Posternak found six equivalents.)

The salt crystallizing with $44H_2O$ described by Posternak was also prepared by allowing the solution to crystallize at about 2°, filtering on an ice-cold funnel, washing with cold water and drying on an ice-cold porous tile. This salt readily redissolves in the mother liquor if allowed to warm up to room temperature before it is dry.

While, therefore, the preparation of sodium phytate obtained from oatmeal is not quite pure, it accords closely in composition and properties with sodium phytate as described by Posternak [1921].

It would have been desirable to use the recrystallized phytate, as prepared for analysis, in the feeding experiments, but the losses were so great that it would not have been possible to prepare the salt in sufficient quantities for feeding dogs. The purified neutral sodium salt precipitated by alcohol as described above was therefore used and was fed to dogs 2644 and 2646. The amount given was equivalent in phytate P to that present in the oatmeal eaten by the animal 2641, half of whose cereal was oatmeal and the other half white flour. For purposes of comparison, one dog (2642) was fed on the same amount of neutral sodium phytate prepared from commercial phytin by the method described in the previous experiment. The phytate from oatmeal was prepared from the same sample of oatmeal as was eaten by 2641.

Basal diet: separated milk powder 20 g. and 6 ml. orange juice added to a mixture of 65 % white flour, 10 % lean meat, 3 % yeast, 14 % cabbage, 7.5 % peanut oil, 1 % NaCl. 80 g. of this mixture were given at the beginning of the experiment and the amount was increased gradually to a maximum of 380 g. The separated milk powder and the orange juice remained unchanged. (Oatmeal contained 0.290 % phytic P.)

Age of puppies at beginning of experiment, 7½ weeks; duration of experiment, 16 weeks.

Results of Exp. 9

No. of puppy	Addition to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur shaft at death A/R ratio	Rickets as judged by X-ray
2641	Cereal: half oatmeal, half white flour	1500	6060	6100	1.11	Moderate rickets
2642	Sodium phytate (from commercial phytin)	1680	5820	6040	1.02	Slight rickets
2643	No addition	1600	6580	6000	1.36	Nearly normal
2644	Sodium phytate (from oatmeal)	1820	6820	6820	1.16	Fairly bad rickets
2645	No addition	2020	7600	7600	1.23	Nearly normal
2646	Sodium phytate (from oatmeal)	1990	7440	7440	1.17	Moderate rickets

In this series of experiments, sodium phytate, whether prepared from commercial phytin or from oatmeal, has made the rickets consistently but only slightly worse and has reduced the Ca in the femur shafts. The interfering effect on calcification was of the same order as that produced by substituting half the white flour of the basal diet by oatmeal (2641).

Exp. 10. The effect of sodium phytate (prepared from oatmeal) on calcification

In view of the smallness in the differences in the degree of rickets in the different dogs in Exp. 9, when oatmeal or sodium phytate was fed at a 50% cereal level, it was thought necessary to attempt to prepare purified sodium phytate from oatmeal in sufficient quantity to add to the basal diet at 100% oatmeal equivalent, i.e. in amount equivalent to that eaten by the animal receiving oatmeal only as cereal (2702), instead of in the half quantities of Exp. 9. The neutral sodium phytate was prepared from oatmeal in the same way as that used in the previous experiment, except that it was necessary to work up the iron phytate in larger batches. The oatmeal eaten by 2702 was defatted, having been through the fat extraction necessary for the first stage in the preparation of the sodium phytate. It belonged to the same batch of oatmeal as that from which the sodium phytate was prepared and contained 0.335% phytic P.

Basal diet: separated milk powder 20 g. and 6 ml. orange juice added to a mixture of white flour 65%, lean meat 10%, cabbage 14%, baker's yeast 3%, peanut oil 7.5%, NaCl 1%. The total of this mixture increased from 100 to 140 g., but the separated milk and the orange juice remained constant throughout. Age at beginning of experiment, 7½ weeks; duration of experiment, 10½ weeks.

Results of Exp. 10

No. of puppy	Additions to diet	Initial wt. (g.)	Final wt. (g.)	Maximum wt. (g.)	Ca in femur	Rickets as judged by X-ray
					shaft at death A/R ratio	
2701	Sodium phytate (from oatmeal)	2120	4300	4580	0.87	Very bad rickets
2702	Defatted oatmeal replaced white flour	2220	5560	5560	0.97	Very bad rickets
2703	No addition	2100	5620	5620	1.20	Slight rickets
2705	Sodium phytate (from oatmeal)	2420	5040	5340	0.97	Very bad rickets
2706	No addition	2520	5960	6120	1.17	Moderate rickets

The larger quantity of sodium phytate will be seen to have produced an intense increase of rickets, reflected both in the radiographs (see Figs. 10–14) and in the Ca content of the femur shaft. As judged by the radiographs and the appearance of the animals (see Figs. 5–9) the degree of rickets produced by the added sodium phytate is comparable with that produced in 2702 by the diet in which defatted oatmeal was the cereal eaten. Judged from the A/R ratios, the degree of rickets produced by sodium phytate at 100% level is slightly worse than that produced by oatmeal. This might be expected, for part of the phytic acid of oatmeal will be combined with Ca. The effect was more definite in the case of the A/R ratios of the humerus bones of these dogs.

DISCUSSION

The experiments described in the preceding part of this paper lead to the following main conclusions: When fed to animals (dogs) receiving a diet with an ordinary Ca:P ratio (a) commercial phytin (CaMg phytate) is not rachitogenic; in fact, it is, if anything, slightly antirachitic; (b) sodium phytate or phytic acid prepared from commercial phytin is strongly rachitogenic, being comparable in potency with oatmeal when fed in a quantity equivalent to the total phytate of the cereal; (c) the sodium phytate fraction prepared from oatmeal retains approximately the full rachitogenic activity of the oatmeal, and this activity is

not diminished by further purification; (d) the rachitogenic activity of sodium phytate is, like that of cereals, counteracted by adding sufficient Ca to the diet.

The first point that emerges from these facts is that the widely accepted view, that the rachitogenic effect of cereals is due to the non-availability of the P of the phytin in the cereals, cannot be true under our experimental conditions. If it were true, phytin and sodium phytate should, in equivalent amounts, produce similar rachitogenic effects, whereas in our experiments on dogs the former is, if anything, slightly antirachitic and the latter produces rickets. (The antirachitic action of phytin has been referred to in earlier publications [Mellanby, 1937; Palmer & Mottram, 1939].) The conclusions of other workers, that the non-availability of phytin P leads to the production of rickets, have been based upon experiments using the unnatural high Ca-low P diet normally employed in studying rickets in rats, and the statement is no doubt true under these conditions. Such conditions bear little relationship, however, to the diets used in our experiments with dogs or to the normal diet of man. Bruce & Callow [1934] attempted to obtain evidence of a rickets-producing action of oatmeal and of phytic acid when fed to rats on a low Ca-high P diet, but these experiments did not give any definite results, doubtless owing to the well-known difficulty of producing rickets in rats on such a diet.

Starkenstein [1914] put forward the view that the toxicity of phytic acid is due to its converting Ca in the body into an un-ionized form. Bruce & Callow drew attention to the insolubility of calcium phytate and suggested that it was possible to assume that, in low Ca diets, phytic acid might exert an anticalcifying action, not on account of the unavailability of the P but by its precipitating action on Ca, which was thus reduced to a deficiency level. While, as pointed out, our experiments do not support the view that the non-availability of phytic acid P has anything to do with the anticalcifying action of cereals under physiological conditions, they do strongly suggest that this anticalcifying effect of cereals is due to the action of phytic acid in rendering Ca unavailable. Indeed, most of the facts established by our experiments appear to receive explanation on this theory. Our view is that the phytic acid in a rachitogenic cereal like oatmeal immobilizes all, or almost all, of the Ca contained in the cereal by converting it into an insoluble Ca phytate which cannot be absorbed, and further, that the excess of phytic acid (over and above that required to precipitate the Ca of the cereal) can exert an additional anticalcifying effect by precipitating further amounts of Ca in the non-cereal part of the diet.

At first sight it might be argued, on the generally accepted view that the phytic acid of cereals is present in the cereal as the CaMg salt, phytin, that our experiments with sodium phytate do not bear any relation to the effect of feeding cereals. For since, as shown in our experiments, phytin itself has not a rickets-producing action, it is clear that the rachitogenic action of cereals cannot be ascribed to their phytic acid content, if all that phytic acid is present as phytin. It can be shown, however, both by calculation and by experiment that such is not actually the case, at any rate as regards oatmeal.

To obtain evidence on this point, a number of samples of the oatmeal used in the feeding experiments were extracted with HCl (10 g. oatmeal, defatted to facilitate extraction, 200 ml. *N*/2 HCl) by shaking for 2 hr. as in the method of McCance & Widdowson [1935] for phytin estimation. The extract was centrifuged and filtered and an aliquot part was then carefully neutralized to pH 7.0 (in a few cases to pH 6.5 or 8.0) by stirring in conc. NaOH drop by drop. After standing for 2 hr. to allow maximum precipitation, the precipitate was centrifuged down, and the amount of Ca and phytate P in the unwashed precipitate

and the phytate P in the supernatant fluid were determined. (The Ca was determined by wet ashing of an aliquot part of the precipitate dissolved in dilute HCl, followed by precipitation and titration as oxalate. The Mg was determined by precipitation as $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, followed by gravimetric or colorimetric estimation. The phytic P was determined by precipitation as ferric phytate by McCance & Widdowson's method.) The figures obtained were somewhat variable, but it was found (a) that nearly all the Ca in the defatted oatmeal was extracted by the HCl, values of 60–70 mg. Ca per 100 g. oatmeal being obtained; (b) that the greater part of this Ca comes down in the precipitate obtained by neutralizing the extract; (c) that the supernatant liquid still contains a considerable proportion of the phytate of the oatmeal, and it can be demonstrated that it is capable of precipitating further quantities of added Ca. The average values for phytate P from one sample of oatmeal were 177 mg. per 100 g. oatmeal in the precipitate and 127 mg. per 100 g. in the supernatant fluid. At pH 8.0 similar values were obtained, while at pH 6.5 precipitation was less complete. The results showed that the ratio of Ca to phytate P in the precipitate (usually about 1:3) is considerably smaller than is the case in commercial phytin. The usual figures for commercial phytin are Ca 12 %, Mg 1.5 %, P 22 %. Much of the phytate precipitated from the oatmeal extract was found to be combined with Mg, and analysis of one sample of unwashed precipitate gave the values Ca 56 mg. and Mg 85 mg. per 100 g. oatmeal. In another experiment in which the pH 7.0 precipitate and supernatant solution were analysed more completely, the following approximate figures were obtained:

	mg. per 100 g. defatted oatmeal	
	Precipitate	Supernatant solution
Phytic P	122	187
Total P	135	—
Inorganic P	Negligible	—
Ca	51	20
Mg	52	103

In this case, the relative amount of phytate, some 60 %, remaining in the solution was larger than usual. The conditions in these experiments might perhaps be taken to represent very crudely the processes taking place in the natural digestion of oatmeal, namely, extraction by HCl (more dilute under natural conditions, but assisted by pepsin), followed by neutralization in the intestine. These experiments suggest that, under such conditions, much of the phytic acid of the oatmeal would be precipitated as phytin, but that this would contain a considerably smaller proportion of Ca than ordinary phytin. Unlike commercial phytin, such a compound might exert some rachitogenic action by exchanging some of its Mg for Ca from the rest of the diet. The number of possible salts of inositolhexaphosphoric acid with more than one metal is very large, and the particular salt which is precipitated from a solution containing the metals will no doubt be determined partly by factors such as pH and the relative concentrations of the bases in the solution. For example, Boutwell [1917] describes the isolation from wheat bran of a phytin with 3.65 % Ca and 10.81 % Mg, compared with 12 % Ca and 1.5 % Mg for ordinary commercial phytin. In the experiments on feeding the Ca-rich commercial phytin, an interchange of metals in the opposite direction may possibly occur to some extent, some of the Ca being set free and replaced by Mg or other bases. Such liberated Ca then perhaps brings about a slightly antirachitic action such as appears to be exerted by commercial phytin.

Apart from this phytate which is precipitated on neutralizing an acid extract of oatmeal, however, there is some 40 % or more of the oatmeal phytate still present in solution and capable of precipitating Ca from other sources. It is true that the supernatant liquid still contains a small amount of Ca in solution, but this Ca may well be in an un-ionized, non-absorbable form. By adding a few drops of CaCl_2 and carefully adjusting to pH 7.0, it is easy to show that the solution is capable of precipitating further amounts of Ca.

Apart altogether from the above experiment, it could have been predicted from the chemical analysis of oatmeal that the phytate is not all present as ordinary phytin. Oatmeal contains about 63 mg. Ca per 100 g., and the average phytate P of our un-defatted oatmeal samples was 253 mg. per 100 g. On the basis of the composition of ordinary phytin given above, more than half the phytate would be present in combination with bases other than Ca, probably other metals such as Na, K or Mg, or possibly with protein [see Lindenbaum, 1926; Mnich, 1931]. The observation that ordinary phytin is not rachitogenic is not then inconsistent with the view put forward in this paper that the rickets-producing action of cereals is due to the phytic acid which they contain. This phytic acid may under natural conditions be reasonably expected to interfere with Ca absorption either by actual precipitation of Ca or by otherwise reducing its ionization and diffusibility. The Ca affected may come partly from the cereal itself—as it were, a passive rachitogenic effect of the cereal—and partly from other foods—an active cereal rachitogenic effect.

This view that the action of cereals is due to interference with Ca absorption accords with the observation that the effect can be prevented by feeding extra Ca, in other words by saturating the phytic acid and rendering it inactive. It has been abundantly proved that the addition of a Ca salt such as calcium carbonate or phosphate prevents oatmeal from having its rickets-producing effect [Mellanby, 1925; Mellanby, 1929]. Similar results were obtained by Palmer & Mottram [1937; 1939] using calcium lactate, and the absence of rachitogenic effect in our experiments with phytin itself again confirms the fact that Ca counteracts the rachitogenic action of phytic acid.

Incidentally, this theory might explain the observation of Lowe & Steenbock [1936, 1] that addition of calcium carbonate to the diet diminishes the hydrolysis of phytate in the intestine of the rat. The extra Ca in the diet would, on our view, result in the precipitation of the whole of the phytic acid as the Ca salt, and it would be expected that this insoluble salt would be attacked by the intestinal flora much less readily than the soluble phytates.

Further, the fact that some foods containing phytin show no rickets-producing action, and the observation that in cereals there is no direct relationship between the amount of phytin P and the rachitogenic action [Harris & Bunker, 1935] is understandable, for the anticacifying action of a foodstuff will depend not merely on how much phytate P it contains but also on how little Ca is present.

Finally, the question must be considered as to what evidence there is that the rachitogenic activity of oatmeal, which we have shown to be retained in the phytic acid fraction prepared from oatmeal, is actually due to the phytic acid itself and not to some impurity in the preparations, for it cannot be claimed that these preparations as fed to the dogs were completely pure sodium phytate or phytic acid, even though the analysis and properties of the crystalline alkaline sodium salt, which we prepared from the solutions used in the feeding experiments, agreed closely with those of pure sodium phytate. The evidence that the phytate is the active factor seems very strong, however, for the following reasons: (a) the sodium phytate fraction from oatmeal produces a rachitogenic effect of a

similar order to that produced by an equivalent amount of oatmeal itself; (b) no loss of activity of the phytate fraction is apparent after further purification; (c) sodium phytate prepared from commercial phytin produces a similar effect; (d) the rachitogenic effect of the phytate fraction is antagonized by feeding extra Ca, which metal is known to precipitate phytic acid; similarly, the activity of oatmeal itself is counteracted by Ca; (e) treatments of cereals which are known to destroy phytic acid, such as boiling with acid or digestion by phytase (e.g. in the autolysis of germinated cereals), lead to a disappearance of rachitogenic activity, whereas the activity is not impaired by treatments such as boiling with alkali, which do not break down phytic acid. It should be mentioned, too, that Anderson [1914] was able to isolate practically pure salts of phytic acid from oats, though the purification involved many stages and the resulting yield was small.

Our experiments do not completely exclude the possibility that there may be other rachitogenic factors in cereals, possibly other inositol phosphoric esters for example, but there appears to be no clear evidence for this at present. Our experiments, however, on the effect of boiling with HCl on the rachitogenic action of oatmeal do seem to indicate that a partial destruction of activity may be brought about by the acid more rapidly than would be expected from the rate of hydrolysis of phytate. A similar conclusion was reached by Lowe & Steenbock [1936, 2] in their experiments with maize, so that the possibility of other rachitogenic factors in cereals cannot be excluded. In our view, the rachitogenic action of cereals is due, at any rate largely, to their content of phytic acid, and this phytic acid acts firstly by immobilizing and preventing absorption of the Ca of the cereal itself and secondly by precipitating or otherwise preventing absorption of further amounts of Ca from the rest of the diet. According to this view, the degree of active interference with calcification produced by a given cereal will depend on how much phytic acid and how little Ca it contains.

The question as to whether phytic acid prevents the absorption of Ca by actually precipitating it as calcium phytate or whether it acts by lowering the amount of ionized or diffusible Ca cannot at present be answered. We have shown that most of the Ca of a dilute HCl extract of oatmeal is precipitated by the phytic acid at neutrality. It is not possible to say whether such actual precipitation occurs under the conditions present in the gut, but it seems not unlikely.

From the point of view of practical human nutrition, the experiments described in this paper show clearly that the rickets-producing action of cereals is to be overcome, not by increasing the P of the diet (as would be the case if the widely held view were true that the cereal action is due to unavailable P), but by increasing the Ca intake, by drinking more milk for example. It appears from these experiments that the rachitogenic action of cereals is only likely to become operative in diets which are on, or below, the borderline of minimum requirements of Ca and vitamin D. It is unfortunate that, for economic reasons, these borderline diets are the ones which so often contain a disproportionately high amount of cereal.

SUMMARY

In experiments made to determine the constituent of oatmeal responsible for its rickets-producing effect, it was found that

(a) phytic acid (inositolhexaphosphoric acid) and neutral sodium phytate prepared from commercial phytin exert powerful rickets-producing actions when added to a non-rachitogenic or slightly rachitogenic diet;

(b) the degree of rachitogenic activity shown by these compounds is roughly comparable with that shown by oatmeal when fed in an amount equivalent as regards phytic acid P;

(c) the phytic acid P fraction extracted from oatmeal itself shows the same rachitogenic action, and purified neutral sodium phytate prepared from this fraction is equally potent;

(d) the rachitogenic action of sodium phytate, as of cereals, is antagonized by adding extra Ca to the diet, and commercial phytin (CaMg phytate) is slightly antirachitic.

Evidence is given suggesting that the rachitogenic action of cereals is normally due not, as has often been suggested, to the unavailability of their P, but to the action of the cereal phytic acid in inhibiting the absorption of Ca from the alimentary canal.

The amount of phytic acid in oatmeal is approximately twice that required to precipitate the Ca of the cereal at neutrality, and it is suggested that the phytic acid exerts its rachitogenic action by preventing absorption both of the Ca of the cereal itself and of further amounts of Ca from the rest of the diet.

We wish to express our thanks to Miss A. S. Frith, who assisted in the care of the animals and preparation of the diets, and to Dr J. R. Hawthorne and Mr W. Buick for much help in connexion with the analyses. We are also grateful to Messrs Parke Davis and Company for generous gifts of takadiastase.

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EXPLANATION OF PLATE II

Experiment 8

Radiographs of wrist bones of dogs after 14 weeks of diet.

Fig. 1. 2637. White flour.

Fig. 2. 2638. White flour.

Fig. 3. 2639. White flour and sodium phytate (from commercial phytin) \equiv 50 % of oatmeal.

Fig. 4. 2640. White flour and sodium phytate (from commercial phytin) \equiv 50 % of oatmeal.

Experiment 10

Radiographs of wrist bones and photographs of dogs after 10½ weeks of diet.

Fig. 5. 2703. White flour.

Fig. 6. 2706. White flour.

Fig. 7. 2701. White flour and sodium phytate (from oatmeal).

Fig. 8. 2705. White flour and sodium phytate (from oatmeal).

Fig. 9. 2702. Oatmeal (defatted).

Fig. 10. 2703. White flour.

Fig. 11. 2706. White flour.

Fig. 12. 2701. White flour and sodium phytate (from oatmeal).

Fig. 13. 2705. White flour and sodium phytate (from oatmeal).

Fig. 14. 2702. Oatmeal (defatted).

For details of experiments, see text, pp. 1671 and 1674.



Fig. 1.



Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.



Fig. 6.



Fig. 7.



Fig. 8.



Fig. 9.



Fig. 10.



Fig. 11.



Fig. 12.



Fig. 13.



Fig. 14.

CCIX. OBSERVATIONS ON THE LIVER FILTRATE FACTOR OF THE VITAMIN B₂ COMPLEX

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WE have been engaged for some time on the purification of the liver filtrate factor of the vitamin B₂ complex [Edgar *et al.* 1938, 2] and in view of recent interesting publications [Hoffer & Reichstein, 1939; Subbarow & Hitchings, 1939; Woolley *et al.* 1939] dealing with obviously closely related factors we desire in the interest of clarity to place on record this interim report of our own experiments.

Our recent studies show that at least three unknown members of the vitamin B complex are required for normal growth by rats receiving the three already identified members, aneurin, riboflavin and vitamin B₆ [Edgar *et al.* 1938, 3; El Sadr *et al.* 1939]. One of these is our liver filtrate factor which can be extracted from acidified aqueous liver extracts by amyl alcohol, but is not adsorbed by fuller's earth; a second factor is neither adsorbable by fuller's earth nor extractable by amyl alcohol and is therefore contained in the residue obtained by successive treatment of liver extract with fuller's earth and amyl alcohol; the existence of at least one further factor is proved by the inferiority of the growth response of rats to optimal amounts of both the above fractions compared with that which results from administration of unfractionated liver extract.

The liver filtrate factor is unstable both to acids and alkalis, especially at high temperatures. It is neither adsorbed nor destroyed by treatment with fuller's earth at pH 1-3; it is, however, adsorbed by relatively large amounts of norite charcoal, from which it can then be eluted by cold aqueous NaOH. Treatment of preparations of the factor from liver with salts of lead, silver and mercury yields bulky precipitates, but the activity remains in the filtrates.

The factor is lacking in any definite basic properties, as shown by its failure to be precipitated from aqueous solution by phosphotungstic acid. It shows a tendency to be adsorbed from alcoholic solutions by inert materials, which probably accounts for the appearance of the major portion of the activity in the precipitate when concentrates of the factor previously neutralized with NaOH are treated in alcoholic solution with phosphotungstic acid. The same explanation may account for the precipitation of the factor from alcoholic solution by barium hydroxide.

Transference of the factor from aqueous solution to amyl alcohol takes place at acid reactions and is reversed by the presence of alkali, indicating that we are dealing with an acid. The neutral methyl ester given by the action of diazomethane is only slightly less active biologically than the free acid, which can be recovered completely by hydrolysis with dilute aqueous Na₂CO₃. The factor is also extracted from acidified solutions by ether, but even after continuous extraction for 43 hr. about half of the activity remains in the aqueous layer. Much inactive material can be precipitated by addition of ether to certain alcoholic concentrates of the

factor; when ether is replaced by ethyl acetate most of the activity remains in solution, but some is carried down with the precipitate. The factor is sparingly soluble in chloroform, for if such ether-precipitated concentrates are treated with that solvent almost all the material and most of the activity pass into solution, but the small residue remaining shows a very high degree of potency; in this way we have obtained material active in daily doses of 180 μ g. per rat. The hydrophilic nature of the factor is shown by its failure to separate from aqueous solution in the form of a salt with either quinine or brucine.

The liver filtrate factor readily yields a chloroform-soluble acetyl derivative which shows only slight biological activity, but which is hydrolysed smoothly by alcoholic ammonia with but little loss of activity. The presence of hydroxy groups is also suggested by the action of acetone. According to the conditions (dryness, pH) this solvent causes more or less complete inactivation of the factor, strongly suggesting the formation of the acetone compound of a glycol.

These properties fit very closely with those described for pantothenic acid by Williams and his collaborators [Williams *et al.* 1938; 1939] and for the chick anti-dermatitis factor by Woolley *et al.* [1938]. Jukes [1939] has already shown that these two substances are probably identical, and Subbarow & Hitchings [1939] have suggested that pantothenic acid is one of the substances present in liver concentrates which influences the growth of rats. We consider that this is very likely, though it should be remembered that the pantothenic acid tested by the above workers may not have approached homogeneity, since it was apparently required in a very much higher dose (8 mg. per rat per week) than that we have found necessary with our best preparations (1.2 mg. per rat per week).

The relationships with "factor W", further properties of which were recently reported on by Frost & Elvehjem [1939], are not clear, and it seems likely that these workers were dealing with a mixture of nutritive factors. Some of the properties they report tally well with those of our liver filtrate factor, whilst others, such as the failure to be extracted with ether from acid solution and the failure to yield a hydrolysable acetyl derivative, virtually exclude the identity. The above authors also claim that concentrates of the chick anti-dermatitis factor have been prepared which show no growth-stimulating action on the rat; this appears to be in direct contradiction to the conclusions of Subbarow & Hitchings [1939]. We are at present engaged in testing the identity or otherwise of our liver filtrate factor with pantothenic acid.

EXPERIMENTAL

The rat growth method for the determination of filtrate factor previously described [Edgar *et al.* 1938, 1] was employed. In our previous work we added the test dose of filtrate factor to the diet of the rats after they had received aneurin, riboflavin and the vitamin B₆ concentrate for 2 weeks. We now find, however, that better results are obtained when the running out period is continued until the rate of weight increase is 7 g. weekly or less; this generally occurs during the third week. Animals given an active dose of the filtrate factor then increase in weight at the rate of approximately 20 g. weekly, which rate is maintained during the 2-week test period. Negative control animals continue to increase in weight at the rate of about 6 g. weekly. Animals which have been deprived of filtrate factor for many weeks respond satisfactorily to added doses of that factor and therefore we have been able to use for further tests animals which had previously acted as negative controls or had received inert fractions; thereby we have effected a saving in animals. Very regular growth responses have resulted on administration of our filtrate factor concentrates and reliable

results have been obtained when only 2 animals were used in each test; nevertheless, we have generally used 3, 4 or more animals to test each fraction. Since we employed such a small number of animals for each test, strictly quantitative results were impossible; we consider, however, that our results were roughly quantitative and that our experimental error was not more than $\pm 25\%$.

Where practicable we have stated the dry weights of our concentrates, together with the number of rat day doses they contained; however, it is not easy to free the concentrates from water and therefore the dry weights are only approximate. To indicate the losses involved in the various fractionations we have also stated the amount of original fresh liver tissue which has yielded that amount of concentrate containing 1 rat day dose of vitamin.

Preparation of liver filtrate concentrates from liver residue III

A convenient source of liver filtrate factor was found in the liver residue previously named residue III [Edgar *et al.* 1938, 2]. This material, which is that portion of an aqueous extract of liver extractable by phenol and not adsorbed by small amounts of charcoal, contains about one-third of the amount of the filtrate factor present in whole liver extract. All evaporations were carried out *in vacuo* below 40° .

3 l. of liver residue III from 500 kg. of fresh liver, adjusted to pH 2 by the addition of H_2SO_4 , were stirred for 30 min. with 500 g. of fuller's earth ("specially selected, activated", Fuller's Earth Union) and filtered; the fuller's earth treatment was repeated. The filtrate (pH 2) was extracted with 8 l. of amyl alcohol; the extract was in turn extracted first with 1 l. H_2O to which was added just enough NaOH to make the aqueous layer alkaline to thymol blue, and then with 500 ml. H_2O . The whole process was repeated ten times using the same amyl alcohol. The combined aqueous extracts from the amyl alcohol, which had been adjusted to pH 8 with H_2SO_4 immediately on separation, were evaporated to 2 l. This fraction contained about 25,000 rat day doses of liver filtrate factor.

A slight excess of a hot solution of basic lead acetate (1200 g. in 3 l. H_2O) was now added to the above material and after stirring and standing overnight in the cold, the copious precipitate, which contained less than one-sixteenth part of the activity, was removed by filtration. The bulk of the lead was removed from the filtrate by addition of H_2SO_4 , the filtrate from the PbSO_4 was adjusted to pH 7 with NaOH and then treated with an excess of a solution of mercuric acetate (40 g. in 250 ml. H_2O) and the inert precipitate filtered off. Excess Hg was removed from the filtrate by H_2S and the filtrate from the HgS evaporated to 1 l. To this solution, adjusted accurately to pH 3 by the addition of H_2SO_4 , 4 l. of 96% alcohol were added and, after standing overnight in the cold, the precipitated salts (mainly Na_2SO_4) were filtered off. The filtrate was evaporated and, when nothing further distilled, about 200 ml. of absolute alcohol were added and the solution again evaporated; this treatment with absolute alcohol followed by evaporation was repeated three times to remove acetic acid and water.

The final gummy concentrate (concentrate A) weighed approximately 70 g. and contained about 20,000 rat day doses of liver filtrate factor; the rat day dose was therefore associated with about 3.5 mg. of dry matter and was contained in the material from approximately 25 g. fresh liver. This method of concentration has been repeated five times and the final material has been reasonably constant in weight and activity.

Much inactive material could be removed from concentrate A by precipitation with phosphotungstic acid in 5% H_2SO_4 , or better by filtration at pH 1 through a column of fuller's earth (1250 g.), the column being washed with water. The

filtrate, which was promptly neutralized with barium hydroxide, contained practically all the original activity and on evaporation yielded a product of which 1.7 mg. = 1 rat day dose (concentrate B). When filtration through fuller's earth is employed, the initial treatment of liver residue III with fuller's earth may be dispensed with.

Stability to acid. To 1.6 g. of concentrate A, 5 ml. H_2O and 5 ml. 20 % H_2SO_4 (by weight) were added and the mixture was heated at 100° for 2 hr. The neutralized material when fed to rats in daily amounts equivalent to 96 g. fresh liver evoked no growth response.

Stability to alkali. Concentrate A was heated in the same manner as above in the presence of 5 % NaOH instead of 10 % H_2SO_4 . There was no demonstrable activity in the material thus treated when fed at the level of 48 g. fresh liver daily.

Adsorption with norite charcoal. To 20 ml. of a solution of the amyl alcohol extract of liver residue III (1 ml. = 240 g. fresh liver containing 12 rat day doses) at pH 2.5, 1 g. of norite charcoal was added. After 30 min. the norite was filtered off and washed with N/100 HCl; the adsorption was repeated a further three times with 1 g. portions of norite. The combined adsorbates were eluted first overnight with 100 ml. 1 % NaOH, then with 50 ml. 1 % NaOH. The eluates were neutralized with HCl. The filtrate from the norite contained the rat day dose in the material from 60 g. of fresh liver and therefore about two-thirds of the activity had been adsorbed. The eluates were active at a level equivalent to 48 g. fresh liver and therefore contained about 42 % of the original activity.

Precipitation with barium hydroxide in alcohol. To a solution of 70 g. concentrate A in 4 l. absolute alcohol, 120 g. of $Ba(OH)_2 \cdot 8H_2O$ dissolved in the minimum amount of hot water were added gradually with very rapid stirring. The final concentration of alcohol in the solution, allowing for the water of crystallization contained in the barium hydroxide, was 96.5 %. The precipitate was filtered off after 16 hr. at 0° and suspended in 500 ml. water in which it partially dissolved. 2 l. 96 % alcohol were then added and after standing in the cold for a few hours the insoluble barium salts were removed by filtration. The barium was removed from all fractions by balancing out exactly with H_2SO_4 . The fraction, which was insoluble in 96.5 % alcohol but soluble in 77 % alcohol, weighed 18 g. and contained about 10,000 rat day doses of liver filtrate factor (1 rat day dose equivalent to 50 g. liver), i.e. it contained about half the activity of the concentrate from which it was prepared and about one-quarter of the original dry matter. Solutions of this material gave an inactive precipitate with Ag_2SO_4 at pH 7, the filtrate being active. The fraction soluble in 96.5 % alcohol had 2500 rat day doses associated with 38 g. dry matter while that insoluble in 77 % alcohol was completely inactive.

Treatment with alkaloids. Attempts made to obtain the active principle from concentrates of varying potency as crystalline quinine or brucine salts failed completely whether the acid concentrate was treated with the free alkaloid or the concentrate neutralized by NaOH was treated with the alkaloid salt. Although several alkaloid salts were separated, the activity remained in the solutions.

Precipitation with phosphotungstic acid in alcohol. To a solution in 100 ml. alcohol of 1.63 g. of a preparation of concentrate B (neutralized with NaOH), which was less potent than other preparations of this material and contained the rat day dose associated with 3.94 mg. dry matter, an alcoholic solution of phosphotungstic acid was added till no further precipitation occurred. The bulky precipitate was filtered off, dissolved in water and decomposed with $Ba(OH)_2$. After removing excess Ba with H_2SO_4 the solution was evaporated. The residue

(0.77 g.) contained two-thirds of the activity of the starting material, while the gum (0.89 g.) obtained on working up in similar fashion the filtrate from the phosphotungstic acid precipitate contained only about one-eighth of the original activity. This treatment therefore effected some purification. Concentrate B, if not previously neutralized with NaOH, gave no precipitate with alcoholic phosphotungstic acid and we therefore consider that the active material in the above experiment was probably carried down by adsorption.

Precipitation with acetone. To 1.78 g. of concentrate A, dissolved in 10 ml. absolute alcohol, 110 ml. acetone were added. The precipitate (1.2 g.) contained slightly less than one-quarter of the original activity, while the filtrate on evaporation yielded a residue (0.57 g.) containing about one-quarter of the original activity. This inactivation was not observed when concentrate A was used in the form of its neutral sodium salt.

Precipitation with ethyl acetate. 1 g. of concentrate B containing about 550 rat doses was treated with 100 ml. hot absolute alcohol and, after keeping for some time at 0°, a small amount of undissolved material was removed by filtration. The alcohol was removed by evaporation, the residue taken up in 10 ml. absolute alcohol, cooled to 0° and the resulting precipitate was filtered off and washed with 3 ml. absolute alcohol. The combined precipitates obtained by these treatments weighed 154 mg. and was biologically inactive.

The filtrate from the above was evaporated, the residue taken up in 6 ml. absolute alcohol, 100 ml. ethyl acetate were added and after 3 hr. at 0° the precipitate was filtered off. The precipitate (242 mg.) contained 80 rat day doses while the filtrate (614 mg.) contained 300 rat day doses. Ethyl acetate therefore gave no satisfactory fractionation.

Precipitation with ether. 500 mg. of concentrate B containing about 275 rat day doses were dissolved in 10 ml. absolute alcohol and after cooling 150 ml. dry ether were added. After 30 min. the precipitated material (240 mg.) was filtered off and was found to contain no activity when 3.2 mg. equivalent to 96 g. fresh liver were fed daily to rats.

Fractionation with chloroform. The residue from the filtrate from the preceding experiment was extracted by shaking for 30 min. with 100 ml. chloroform (undried) and the chloroform solution decanted from insoluble material adhering to the sides of the vessel. The chloroform-soluble portion (240 mg.) contained 150 rat day doses, each associated with 1.6 mg. of dry matter, while that insoluble in chloroform (10 mg.) contained 56 rat day doses of filtrate factor. The rat day dose in the latter fraction was therefore associated with only about 180 µg. of solids; however, the loss of activity in the preparation of this fraction was high. In a further experiment in which the chloroform extraction was less thorough about half the activity remained in the chloroform-insoluble fraction but in this case the rat day dose was associated with 400–500 µg. of dry matter.

Effect of hydrogen ion concentration on extractability of liver filtrate factor by amyl alcohol. In the preparation of concentrate A we obtained satisfactory extraction of liver filtrate factor with amyl alcohol at pH 2. In other experiments we extracted crude liver preparations (liver residue I [see Edgar *et al.* 1938, 2]) at various hydrogen ion concentrations with amyl alcohol using a procedure similar to that already described (p. 1683). At pH 1 and pH 3 the activity was extracted satisfactorily but the extract obtained at pH 7 contained only about one-half of the activity present in those obtained by extraction of the acidified solutions.

Extraction with ether from aqueous solution. 4.4 g. of concentrate A dissolved in 20 ml. H₂O adjusted to pH 2.5 with H₂SO₄ were continuously extracted with ether during 15 hr. The ether was changed and the extraction continued for a

further 28 hr. The first ether extract (1.2 g.) contained about one-third of the activity present in the starting materials, the second (250 mg.) about one-eighth and the residue between one-quarter and one-half.

Acetylation experiments. 3.36 g. of concentrate A were taken up in a mixture of 2 ml. pyridine and 50 ml. acetic anhydride, the solution was heated on the water bath for 2 hr. and then evaporated. The residue was completely soluble in chloroform and was partitioned between this solvent (100 ml.) and water (100 ml.). Chloroform phase: this was evaporated to dryness and a portion (one-third) heated on the water bath for 30 min. with 100 ml. water to which had been added 5 ml. of 3*N* HCl. The resulting solution on evaporation yielded a gum which had little or no biological activity (inactive in amount = 96 g. fresh liver). The remaining two-thirds of the material from the chloroform phase was hydrolysed by heating at 100° in a sealed tube with alcoholic ammonia (saturated at 0°). The solvent and excess ammonia were removed and the residue freed from acetamide by extracting its aqueous solution with chloroform. Evaporation of the aqueous layer gave a brownish syrup (0.8 g.) which was active when fed to rats at a level equivalent to 48 g. original liver. Aqueous phase: this was hydrolysed with alcoholic ammonia at room temperature for 48 hr. and gave a residue fully active at a dosage equivalent to 96 g. fresh liver.

In another acetylation experiment with concentrate A the acetylated product was hydrolysed using sodium methoxide according to the method of Zemplén & Pácsu [1929]. The regenerated material was fully active when fed at a level equivalent to 48 g. fresh liver (i.e. 75 % activity recovered in first experiment and at least 50 % in second).

The unhydrolysed acetyl fraction when fed in an amount equivalent to 48 g. of the original liver evoked only a very slight response and twice this amount also produced a suboptimal response (average weekly weight increase of 8.8 g. and 15 g. respectively, compared with 6 g. weekly for negative control rats and 23 g. weekly for rats receiving the hydrolysed material equivalent to 48 g. original liver daily). Whether this activity is due to unacetylated material or whether the acetyl derivative actually possesses slight biological activity is not known.

Treatment with diazomethane. 1 g. of concentrate B in 50 ml. absolute alcohol was treated with an excess of diazomethane in ethyl alcohol and allowed to stand for 30 min.; the solution was then neutral to litmus. The solvent was removed and the residue taken up in 20 ml. absolute alcohol. One-half of this material was heated at 30° for 2 hr. with 100 ml. *N*/20 Na₂CO₃, the solution cooled, neutralized with H₂SO₄ and evaporated. Both the hydrolysed and unhydrolysed portions when fed to rats at levels equivalent to 48 g. original liver evoked growth responses. The rate of weight increase produced by the hydrolysed material was about 18 g. weekly; the unhydrolysed material produced growth rates averaging 16.5 g. weekly. This difference may not be significant.

SUMMARY

1. Liver filtrate factor is not precipitated by salts of lead, mercury, silver, quinine or brucine, but is precipitated from alcoholic solution by barium hydroxide.

2. The factor is not adsorbed even on exhaustive treatment with fuller's earth; it is, however, adsorbed by large amounts of norite charcoal. Amyl alcohol and ether extract the factor from acidified solutions.

3. By combination of several methods of concentration a material has been obtained containing the rat day dose of the vitamin associated with about 180 µg. of dry matter.

4. On acetylation liver filtrate factor yields a product easily extractable from aqueous solution by chloroform and possessing only feeble activity; mild hydrolysis of this material gives a product having biological activity comparable with that of the starting material. Treatment of a liver filtrate factor concentrate with diazomethane does not appreciably affect the biological activity.

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CCX. THE EFFECT OF ADMINISTRATION OF AN ACETATE ON DETOXICATION AND THERAPEUTIC ACTIVITY OF SULPHANILAMIDE

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ONE of the means by which the animal body protects itself against foreign toxic substances is acetylation as illustrated by the conversion of aniline into acetanilide [Englehart, 1888], and by the acetylation of *m*- and *p*-aminobenzoic acids [Sherwin *et al.* 1926]. Since acetylation also occurs after the administration of sulphanilamide [Marshall *et al.* 1937] it became of interest to enquire whether acetylation would diminish the toxicity of sulphanilamide without affecting its curative value.

The experiments here reported deal with this. Following the lead of other workers [Knoop, 1910; 1911; Hensel, 1915; Sherwin *et al.* 1927; Klein & Harris, 1938], who showed that the acetyl group may be derived from acetates, lactates or pyruvates, sodium acetate was first administered along with sulphanilamide in order to determine whether this would increase the amount of acetyl-sulphanilamide eliminated.

Analytical methods

Sulphanilamide and acetylsulphanilamide were estimated in the mouse urines by the method of Fuller [1937]. For ease of comparison, acetylsulphanilamide has been calculated in terms of sulphanilamide throughout this paper.

The urines were examined for *p*-aminophenol by the indophenol reaction, and the hydroxylamine compound was estimated by the method of Pucher & Day [1926]. *p*-Aminophenol and *p*-hydroxylaminobenzenesulphonamide have been found in the urine of patients treated with sulphanilamide and may be responsible for some of the toxic symptoms; a paper dealing with this subject is in course of preparation.

Table I

	mg./100 ml. free sulphanilamide			mg./100 ml. acetyl- sulphanilamide		
	Actual	Found	% recovery	Actual	Found	% recovery
Unheated urine:						
Decolored	2	2.04	102			
	4	3.92	98			
	8	8.3	104			
Heated urine:						
Decolored	5	4.2	84	5	4.8	96
	10	10.1	101	10	10.6	106
Diluted 25 times	5	2.6	52	5	3.6	72
	10	8.8	88	10	9.2	92

¹ Working under a full-time grant from the Medical Research Council.

Preliminary experiments on the recovery of acetylsulphanilamide from mouse urine gave low results after hydrolysing with 2N HCl; the results were not improved by dilution but, as Table I shows, decoloring with zinc hydroxide gave better values.

The actual method adopted was to add 2 ml. of 10 % ZnSO_4 to each 5 ml. heated urine, adjust to pH 7-8 by addition of 10 % ammonia and dilute the whole to 10 ml. or other suitable volume, filter and take aliquots of the filtrate for the estimations.

Effect of acetate on production of acetylsulphanilamide

Two groups of 10 mice were used in this experiment which was done to ascertain the effect, if any, that simultaneous dosing with sodium acetate (crystalline) would have on the production of acetylsulphanilamide.

Group A was given 10 mg. sulphanilamide per mouse orally on each of 4 days; then after 3 days with no treatment these mice were each given 10 mg. sodium acetate and 10 mg. sulphanilamide for a further period of 4 days. This was followed by 3 days' rest, at the end of which the mice were killed. During these periods the urine was collected for analysis.

Group B received 10 mg. sulphanilamide and 10 mg. sodium acetate per mouse for the first period of 4 days; 3 days' rest followed, dosage being then continued with 10 mg. sulphanilamide for 4 days and 3 days' rest before killing. As before the urines were collected and analysed. The detailed results are shown in Table II.

Table II

Period	Dose	Group A				Dose	Group B			
		Hydroxyl-amine compound mg.	Sulphanil-amide				Hydroxyl-amine compound mg.	Sulphanil-amide		
			Free mg.	Acetyl mg.	Ratio A/F			Free mg.	Acetyl mg.	Ratio A/F
1st 4 days	Sulph.	6.7	27.4	15.6	0.6	Sulph. + acetate	1.7	24.8	27.2	1.1
2nd 3 days	None	2.3	20.0	13.4	0.7	None	0.0	10.0	5.0	0.5
3rd 4 days	Sulph. + acetate	2.7	23.5	39.5	1.8	Sulph.	2.0	15.8	3.4	0.2
4th 3 days	None	0.8	17.2	9.5	0.6	None	0.9	11.0	5.0	0.5

Table III

		Group A							Group B				
Period	Dose	Amino-phenol	Hydroxyl-amine compound mg.	Sulphanil-amide			Dose	Amino-phenol	Hydroxyl-amine compound mg.	Sulphanil-amide			
				Free mg./100 ml.	Acetyl mg./100 ml.	Ratio A/F				Free mg./100 ml.	Acetyl mg./100 ml.	Ratio A/F	
1st 4 days	Sulph.	+	2.8	50.0	25.3	0.5	Sulph. + acetate	-	2.5	35.5	21.4	0.7	
2nd 8 days	None	++	8.3	10.0	5.2	0.5	None	-	6.8	14.0	11.7	0.8	
3rd 4 days	Sulph. + acetate	-	1.8	102.0	76.0	0.7	Sulph.	-	1.7	125.0	25.5	0.2	
4th 8 days	None	+	7.5	20.0	8.0	0.4	None	+	3.3	15.1	2.5	0.2	

No *p*-aminophenol was detected in any of the urine specimens of Table II although the hydroxylamine compound was present. Examination of human urine has shown that the oxidation products of sulphanilamide are excreted for

some considerable time after cessation of therapy and this probably accounts for the slightly increased excretion of the hydroxylamine compound in the third period with group A which was then receiving acetate in addition to sulphanilamide.

The above cross-over experiment was repeated with two more groups of mice; this time each group containing 8 mice. The amount of acetate was halved but was still more than the theoretical amount necessary entirely to acetylate the sulphanilamide; also the period of rest between doses was increased to 8 days. Table III shows these results.

Tables II and III are summarized in Table IV which shows that administration of acetate increases the amount of acetyl derivative formed during its period of administration and in addition may have some effect in lessening the oxidation products, the improvement appearing to depend on the amount of acetate given especially during subsequent resting periods when there is no difference between the total hydroxylamine outputs.

Table IV

Treatment ...	Sulphanilamide mg. excreted			Sulphanilamide + acetate mg. excreted		
	Hydroxyl- amine compound	Free	Acetyl	Hydroxyl- amine compound	Free	Acetyl
Periods of administration:						
4 days	6.7	27.4	15.6	1.7	24.8	27.2
4 days	2.0	15.8	3.4	2.7	23.5	39.5
4 days	2.8	50.0	25.3	2.5	35.5	21.4
4 days	1.7	125.0	25.5	1.8	102.0	76.0
Totals	13.2	218.2	69.8	8.7	185.8	164.1
Ratio A/F			0.31			0.88
Periods of rest:						
3 days	2.3	20.0	13.4	0.0	10.0	5.0
3 days	0.9	11.0	5.0	0.8	17.2	9.5
8 days	8.3	10.0	5.2	6.8	14.0	11.7
8 days	3.3	15.1	2.5	7.5	20.0	3.0
Totals	14.8	56.1	26.1	15.1	61.2	29.2
Ratio A/F			0.47			0.48

Effect of acetate on antistreptococcal activity

The protective effects of sulphanilamide and sulphanilamide with acetate were compared by infecting groups of mice with lethal doses of group A haemolytic streptococcus, reserving one group of mice as controls and giving five small doses (2.5 mg.) of the drug to each of two other groups of mice at intervals over 48 hr., finally calculating the average life of each series by Whitby's method [1937]. The results of five separate experiments are grouped in Table V.

Table V

Strain	Number of mice in each group	Average life of mice in days		
		Controls	Sulphanilamide	Sulphanilamide + acetate
L 193	8	3.8	10.5	13.0
Richards	8	2.0	11.0	11.0
Richards	8	1.8	12.3	12.0
Richards	6	1.8	6.7	4.1
Richards	5	1.7	12.5	12.0
Average		2.2	10.6	10.4

It appears that acetate does not materially reduce the protective power of sulphanilamide and hence its administration may be advantageous if it reduces acute or chronic toxicity.

The effect of acetate on toxicity

Acute toxicity. The acute toxic effects were produced by giving 100 mg. sulphanilamide orally to each mouse in two groups of 5 mice. In addition, in one of these groups each mouse received 100 mg. sodium acetate.

After 2 hr. the mice receiving sulphanilamide were all distressed and three of them showed the spasticity, inco-ordination and hyperventilation described by Hoare [1939]. At the end of 5 hr. a second dose of 50 mg. sulphanilamide was given; this was very shortly followed by a repetition of the previous symptoms and death of 1 mouse within 24 hr., the remainder recovering with time.

The second group of mice dosed with acetate was also given another dose of 50 mg. sulphanilamide and 50 mg. acetate: these mice showed no symptoms of distress throughout the experiment.

A control experiment giving sodium acetate alone showed no ill effects in any of the 5 mice of this group.

The previous observations were confirmed by repeating the experiment with two groups, each of 6 mice, using the same procedure as before. After 2 hr. all the mice which had received sulphanilamide alone were completely prostrate and spastic, breathing deeply. Two died at 26 and 27 hr. respectively; the other four gradually recovered.

The mice which had received acetate as well as sulphanilamide presented a very striking contrast. Apart from some deep breathing they appeared in good condition after 2 hr. Later one became prostrate and died, the others recovering.

Chronic toxic effect. On account of the alkalosis produced by sodium acetate, it was thought that ammonium acetate might be preferable, but a preliminary experiment with this salt, both alone and with sulphanilamide, showed that more rapidly fatal results were obtained, probably owing to the known toxic effects of the ammonium ion.

Six mice were each given 25 mg. of sulphanilamide twice daily, the dose being increased by 5 and 10 mg. increments until a total dosage of 50 mg. was reached. When the total given was 220 mg. these mice became slow in their movements and after a further 50 mg. lack of co-ordination and hyperventilation were observed, but all mice recovered after a period of 20 hr. from the last dose.

The second group of mice received doses of sodium acetate in the same amount as the sulphanilamide and increasing from 25 to 50 mg. When 220 mg. of sulphanilamide had been given two of these mice showed nervous symptoms and hyperventilation. A further dose of 50 mg. was then given, and another two mice displayed nervous symptoms and over-breathing, one dying 20 hr. after the last dose; the others recovering with time.

A control experiment with doses of sodium acetate graduated in the same manner from 25 to 50 mg. showed no ill effects after a total of 300 mg. had been reached, although as would be expected the urine had a high pH (8.2).

To confirm these results another experiment was performed in which the actual food consumption was determined by weighing the food and also each mouse daily throughout the experiment. As before, two groups of mice were used, one group being fed with 50 mg. sulphanilamide per mouse daily whilst the other group received 50 mg. sulphanilamide and 25 mg. sodium acetate daily. The experiment continued for 17 days before the mice were killed and

during this time the body weight changes and food consumption of each group were almost exactly parallel as will be seen by reference to Fig. 1.

After killing, leucocyte and differential counts were performed and no significant difference was found between the two groups.

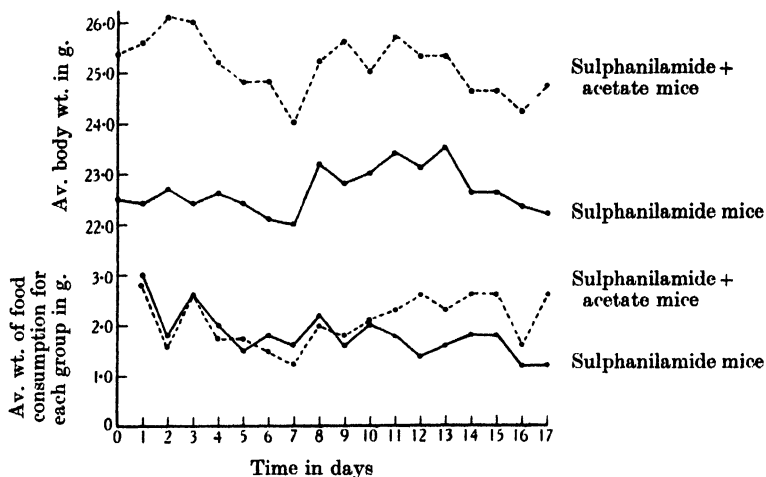


Fig. 1.

Administration of acetylsulphanilamide

Curative effect. The slight antistreptococcal activity mentioned by Buttle *et al.* [1936] and by Tréfouel *et al.* [1937] was confirmed on batches of 6 mice administering 18 mg. of acetylsulphanilamide (equivalent to 15 mg. sulphanilamide) in five equal doses to infected mice over 48 hr.; 15 mg. sulphanilamide were also given in similar doses to another batch of infected mice. The average life of the infected controls was 1.8 days (0 survivors for 14 days), that of the acetyl-treated mice was 2.8 days (0 survivors for 14 days) and of the sulphanilamide-treated mice 6.7 days (2 survivors for 14 days).

Acute toxic effect. Marshall *et al.* [1938], giving doses of 2 g. per kg. (i.e. 40 mg. per 20 g. mouse) to a group of 20 mice, reported 9 deaths (45%); with a similar dose of sulphanilamide to a batch of 28 mice, 2 deaths (7.1%) resulted. The deaths all occurred within 4 days.

Using these same doses with a group of 16 mice, 7 deaths (44%) occurred in the same time, thus confirming the results of Marshall. No deaths resulted from treatment of a group of mice with the same dose of sulphanilamide.

When acetate was given along with the acetylsulphanilamide to 8 mice, there was no significant difference in the results as 4 deaths occurred, a mortality of 50%. In an attempt to avoid the deaths occurring on the 3rd and 4th days after acetyl dosage, the acetate was given in doses of 40 mg. each of the first three successive days, without affecting the result.

DISCUSSION

The observation that increased acetylation of sulphanilamide occurred in mice when that drug was administered along with sodium acetate, and the remarkable effect of such simultaneous administration in reducing the acute toxicity of the sulphanilamide suggest at first sight that acetylation is a de-

toxicating mechanism. This interpretation is, however, difficult to reconcile with the observation of Marshall and his colleagues (confirmed by our own experience) that acetylsulphanilamide when administered as such to mice, is definitely more toxic than the parent sulphanilamide.

In face of this apparent discrepancy it is well to bear in mind that the injurious effects of a drug are not always attributable directly to its toxic affinity for certain tissue cells; they may be due rather to the sudden withdrawal of some body constituent in an attempt to detoxicate the drug by synthesis, or to quite different processes such as a profound physical or chemical alteration of the body fluids or again to blockage of an organ such as the kidney.

In the case under consideration here it seems possible that sulphanilamide kills chiefly by sudden withdrawal from the body of acetyl-precursors. The administration of acetylsulphanilamide as such would not make this sudden demand but may well be injurious in other ways.

The co-administration of acetates or acetate precursors and sulphanilamide to human subjects may be important since it should spare the liver and also reduce the amount of the oxidation products of sulphanilamide which produce various toxic reactions in the human. While this has not yet been attempted, it has been found that the dosing with citrates along with sulphanilamide does have some effect in increasing the amount of acetyl derivative formed.

More work is still to be done on this problem and experiments are in progress which may throw more light on the subject, including a histological examination of the tissues of the animals treated by the methods mentioned above.

SUMMARY

Mice receiving sodium acetate along with sulphanilamide, excreted more acetylsulphanilamide than mice receiving the same dosage of sulphanilamide. Such combined treatment did not diminish the curative value of the drug but was effective in modifying the acute toxic symptoms resulting from a single large dose of sulphanilamide. When smaller doses of sulphanilamide were given over a longer period, acetate did not appreciably affect the results.

My thanks are due to Dr L. Colebrook for performing the animal experiments and I wish to express my gratitude to Dr Fuller and Mr Maxted for many useful suggestions and assistance.

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CCXI. XANTHINE OXIDASE AND MILK FLAVOPROTEIN

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With an Addendum by J. ST L. PHILPOT

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BALL [1938; 1939], in a thorough investigation, isolated a flavoprotein from cow's milk and presented evidence which rendered highly probable its identity with the xanthine-aldehyde oxidase. Corran & Green [1938, 1, 2] simultaneously isolated from the same source a flavoprotein which showed no activity as xanthine-aldehyde oxidase but which could catalyse the oxidation of dihydrocoenzyme I. The recorded properties of the two flavoproteins appeared to be identical except with respect to catalytic activity. The aim of the present investigation was to determine by further purification whether there are two flavoproteins, one catalysing the oxidation of hypoxanthine and aldehyde, the other catalysing the oxidation of dihydrocoenzyme; and if there is in fact only one flavoprotein, to explain why Corran & Green were unable to find any xanthine-oxidase activity in their flavoprotein preparation.

We have succeeded by a new method of preparation in isolating a flavoprotein which acts both as xanthine-aldehyde oxidase and as dihydrocoenzyme I oxidase. By appropriate treatment of the flavoprotein, xanthine-aldehyde oxidase activity can be eliminated completely without any loss of dihydrocoenzyme I activity. Thus the two catalytic activities, although associated with the same flavoprotein, are entirely independent functions of this flavoprotein.

Milk flavoprotein is not simply a protein combined with flavin adenine dinucleotide. In addition to flavin there is some other coloured compound of unknown constitution which accounts for the brownish red appearance and the atypical spectrum of milk flavoprotein. The catalytic role, if any, of this additional coloured group has yet to be clarified. In the absence of this information it is unwise to say definitely that flavin is the prosthetic group of milk flavoprotein in the sense that it is the functional group in the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I.

(1) *Method of testing catalytic activity*

Xanthine oxidase of milk catalyses the direct oxidation of hypoxanthine or xanthine by molecular O_2 [Morgan *et al.* 1922; Dixon & Thurlow, 1924]. With purification of the enzyme the direct reaction with O_2 becomes erratic—the velocity dropping to zero within a few minutes of the initiation. At the highest purity level the reaction at 38° comes to a stop almost at once. Clearly the manometric method is unsuitable for following the activity of the xanthine oxidase in the course of purification. As regards the other substrates, viz. aldehyde and dihydrocoenzyme I, the direct reaction with O_2 is negligible even in the crudest preparations of the enzyme. Addition of methylene blue as carrier increases the

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rate of reaction enormously but even this expedient fails at the higher purity levels to ensure the reliability of the manometric method. Our experience has been that the anaerobic Thunberg method using methylene blue as hydrogen acceptor offers a more satisfactory method of following the activity of the xanthine oxidase.

We have arbitrarily defined as the unit of xanthine oxidase activity an amount of enzyme that in presence of 1 mg. of hypoxanthine will catalyse the reduction of 0.1 ml. of 0.0113*M* methylene blue in 1 min. The experiments were carried out at 38° in a total volume of 2.5 ml. There is a direct proportionality between enzyme concentration and rate of reduction of methylene blue except at low concentrations of enzyme. In practice we have selected for our tests an amount of enzyme which gave a reduction time not greater than 5 min. and not less than 20 sec. Beyond these limits the measurements became unreliable under the conditions of the experiment.

(2) *Method of preparation*

When dealing with a coloured protein having a characteristic absorption band in the visible spectrum the spectrophotometric method offers a quick and reliable measure of the degree of purification attained by a given procedure. The theory of the method is briefly as follows. The light absorption of a flavoprotein at *ca.* 450 $m\mu$ is due to the flavin moiety whereas the absorption of 275 $m\mu$ is due to both the flavin and the protein. Any colourless protein impurity will increase the absorption at 275 $m\mu$ but not at 450 $m\mu$. Thus the ratio $E_{275\text{ }m\mu} : E_{450\text{ }m\mu}$ becomes a measure of the amount of protein impurity in a preparation of flavoprotein. With purification the ratio becomes smaller and approaches a fixed value characteristic of the homogeneous protein. A decrease in the ratio is evidence that purification has been achieved though it is impossible to estimate accurately the decrease in protein impurity from the decrease in the ratio. The light absorption at 275 $m\mu$ is a function of the amino-acid content and it does not follow that the protein impurities absorb equally at this wave-length or even absorb at all. In practice we have found the ratio method to be a reliable index to the progress of purification—agreeing reasonably well with the dry weight method.

The following are the details of the method for preparing the flavoprotein which is associated with xanthine-aldehyde-dihydrocoenzyme I activity and which is *ca.* 1000 times more active per mg. dry weight than an average sample of milk. Some 50 l. of milk have to be processed to ensure adequate material in the final stages of purification.

(1) 57 l. of milk containing 11,000 units were warmed to 30°, saturated with NaCl and filtered through fluted papers (unpublished method of M. Dixon & R. Lemberg). Filtration was complete in 24 hr. The clear yellow filtrate (45 l.) was mixed with an equal volume of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was filtered through fluted papers (time *ca.* 6 hr.). The precipitate was scraped off and dissolved in 750 ml. *M*/10 phosphate buffer *pH* 7.2—final vol. 1270 ml. containing 2780 units. Allowing for the $(\text{NH}_4)_2\text{SO}_4$ introduced by the precipitate the solution was made 40% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged off and redissolved in water—final vol. 820 ml. containing 2300 units. The enzyme solution was deep brown at this stage and somewhat opalescent.

(2) The above solution was dialysed for 2 hr. against running tap water and then submitted to the first $(\text{NH}_4)_2\text{SO}_4$ fractionation. Enough *M*/2 Na_2HPO_4 was added to bring the *pH* to 7.5 and the enzyme solution was then made 35% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$ by slow addition of a saturated solution.

The precipitate was discarded. The supernatant fluid was brought to 45% saturation: the precipitate was centrifuged and dissolved in water, final vol. 223 ml. containing 1270 units.

(3) The above solution was dialysed for 2 hr. against running tap water, cooled to 0° and then mixed with 1/5 vol. *M*/2 acetate buffer *pH* 4.6. Ethyl alcohol was added sufficient to bring the concentration up to 13%. The solution was cooled to -5° and centrifuged at the same temperature. The precipitate was dissolved in *M*/100 phosphate buffer, final vol. 117 ml. containing 1000 units.

(4) The above solution was acidified to *pH* 5.8 with 10% acetic acid. Alumina *Cγ* gel containing 15 mg. per ml. was added in five successive lots (25 ml.) each of which was centrifuged separately. The first two lightly coloured lots were discarded and the final more deeply coloured lots were combined, washed with water and eluted with *M*/2 phosphate buffer *pH* 7.2 until almost colourless. The combined eluates (240 ml.) contained 600 units. The flavoprotein was concentrated by precipitating with 42% saturated $(\text{NH}_4)_2\text{SO}_4$ and re-dissolving in 50 ml. water (550 units). To remove the last traces of alumina the solution was spun hard for 30 min. The supernatant fluid was water-clear and deep brownish red.

(5) The above solution was dialysed against running distilled water for 36 hr. at 3°. The slightly coloured precipitate which was formed was discarded. The clear centrifuged solution was brought to *pH* 7.5 by the addition of *M*/10 Na_2HPO_4 solution and subjected to the second $(\text{NH}_4)_2\text{SO}_4$ fractionation. Saturated $(\text{NH}_4)_2\text{SO}_4$ was added dropwise until a definite turbidity formed. After 15 min. the precipitate was centrifuged. In this way four successive fractions were obtained between the limits 35–45% saturation of $(\text{NH}_4)_2\text{SO}_4$. The first and fourth fractions were the least coloured and were discarded. The second and third fractions were dissolved in water and combined, final vol. 25 ml. containing 300 units. The procedure of dialysis and removal of insoluble material followed by $(\text{NH}_4)_2\text{SO}_4$ fractionation was repeated twice again before the highest purity level was reached. The third and fourth $(\text{NH}_4)_2\text{SO}_4$ fractionations were carried out between the narrower limits of 38 and 45% saturation. We have found no rule governing which of the fractions will have the highest purity level. In practice the most deeply coloured fractions were selected and the correctness of the selection checked by determining the ratio $E_{275\text{ m}\mu} : E_{450\text{ m}\mu}$. The final solution (20 ml.) containing 200 units had an extinction ratio of 6.2. The overall yield from the original milk to this stage was $\frac{200}{11,000} \times 100$ or 1.8%.

At the extinction ratio 6.2 stage 0.60 mg. of the flavoprotein is equivalent to one enzyme unit. In an average sample of cows' milk 5 ml. containing 624 mg. dry weight are equivalent to one enzyme unit. The degree of concentration relative to milk is therefore $\frac{624}{0.60} = 1040$. One l. of milk of average activity would contain 120 mg. of the flavoprotein at the 6.2 extinction ratio stage.

Table I summarizes the various procedures used in the method and the degree of purification obtained by each stage. There is considerable variation from one lot of milk to another in the efficiency of the various purification procedures. The values recorded in the table represent the averages of many experiments.

At the purity level represented by extinction ratio 6.2 the flavoprotein is still not homogeneous. Mr J. Philpot discusses the ultracentrifugal data in the Addendum to this communication. The flavoprotein component accounts for 80 to 83% of the total sedimenting material in the preparation. Repetition of $(\text{NH}_4)_2\text{SO}_4$ fractionation failed to separate the flavoprotein from the persistent

Table I. *Summary of purification procedures*

Stage	Volume ml.	Enzyme units	$E_{275\text{ m}\mu} : E_{450\text{ m}\mu}$
Milk	57,000	11,000	—
$(\text{NH}_4)_2\text{SO}_4$ precipitate of NaCl filtrate	1,270	2,780	—
After 1st $(\text{NH}_4)_2\text{SO}_4$ fractionation	223	1,270	50
After alcohol precipitation	117	1,000	25
After alumina adsorption	50	550	15
After 2nd $(\text{NH}_4)_2\text{SO}_4$ fractionation	25	300	10
After 4th $(\text{NH}_4)_2\text{SO}_4$ fractionation	20	200	6.2

impurity referred to as the γ component in the photograph of the ultracentrifuge run. A selective reagent has yet to be found for the final stages of the purification.

In general the xanthine-aldehyde oxidase activity is strictly proportional to the flavoprotein concentration. The ratio (xanthine-aldehyde activity : flavoprotein concentration) remains constant from the first clear extract which can be assayed to the final stage. However, we have observed particularly in the summer months that the ratio falls off in the early stages of the purification. For example, a neutral dilute salt solution of the flavoprotein at the end of stage 1 of the purification lost 50 % of its xanthine-aldehyde activity after storage for 12 hr. at 0° although the concentration of the flavoprotein remained unaltered. High salt concentrations and low temperatures (-10°) retard this inactivation but we have failed to find any method of stopping it apart from rapid manipulation. The phenomenon was not observed during the autumn and winter months. The inactivating agent appears to be removed after the alumina stage. Flavoprotein preparations of ratio $E_{275\text{ m}\mu} : E_{450\text{ m}\mu} = 12$ or less are stable at room temperature in presence of 5–10 % $(\text{NH}_4)_2\text{SO}_4$ or at 0° in absence of salts. Capryl alcohol can be used as a preservative without any effect on the enzyme.

(3) *Absorption spectrum*

Fig. 1a shows the absorption spectrum of a solution of milk flavoprotein at the extinction ratio 6.2 stage. There are maxima at 275, 350 and at 450–453 $\text{m}\mu$. The same solution and its control were treated with hydrosulphite. The spectrum recorded after reduction is shown on Fig. 1b.

The dry weight¹ of the solution was 2 mg. per ml. On the assumptions (1) that all the absorption at 450 $\text{m}\mu$ is due to the flavin group, and (2) that the absorption coefficient $\beta = 2.4 \times 10^7$ as for other flavoproteins then the lacto-flavinphosphate content of this preparation should be 0.77 %. If the first of the above assumptions were correct we should expect that addition of hydrosulphite would completely eliminate absorption at this wave-length. In fact the hydrosulphite reduced solutions of flavoprotein show about 40 % of the absorption of the oxidized form at 450 $\text{m}\mu$. It would seem, then, that not more than 60 % of the absorption at 450 $\text{m}\mu$ can be due to flavin.

The flavin moiety of milk flavoprotein can replace the flavin adenine dinucleotide coenzyme of the *d*-amino-acid oxidase [Ball, 1939; Corran & Green, 1938, 2]. The method of estimating the dinucleotide by the *d*-amino-acid oxidase test has already been described by Corran *et al.* [1939]. By this method the

¹ The dry weight estimations were carried out as follows. Samples of the test solution which had been dialysed for 5 days against distilled water were frozen and dried in high vacuum over H_2SO_4 for 48 hr. The dry weight was determined in triplicate after moisture equilibrium had been reached. Drying at 100° *in vacuo* led to an 8 % loss in weight. There was also an ash residue of 4.5 % after combustion (Weiler, Oxford). The dry weight was corrected for residue ash and moisture.

amount of dinucleotide present in any given solution of flavoprotein can be determined. The flavin group was split off from the protein by exposing the solution to $N/6$ HCl for 5 min. at room temperature. The solution was then rapidly neutralized by the addition of solid Na_2HPO_4 . The flavin concentration as

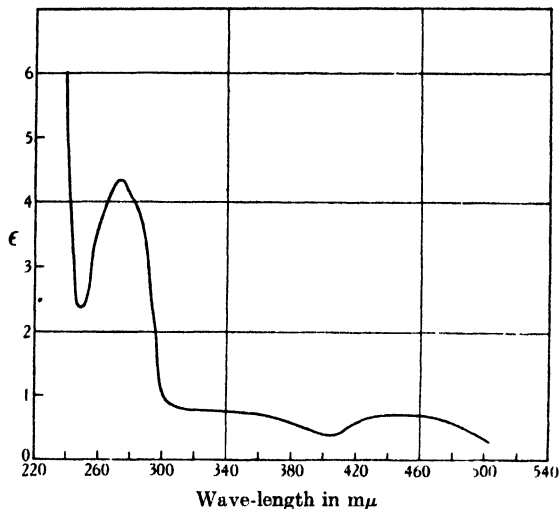


Fig. 1 a.

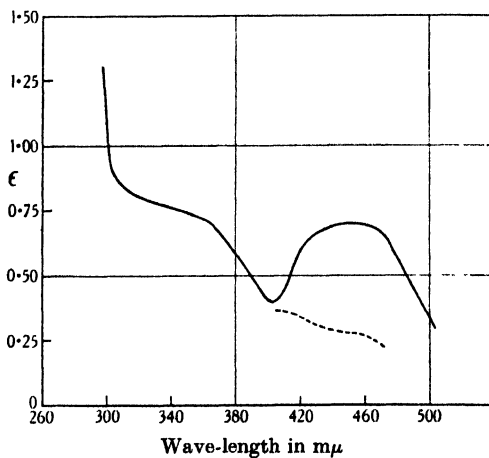


Fig. 1 b.

Fig. 1. (a) Absorption spectrum of 0.2% milk flavoprotein solution (0.77% apparent flavin-phosphate). (b) Comparison of oxidized (—) and hydrosulphite-reduced (----) flavoprotein (0.2%).

determined by the O_2 uptake in the *D*-amino-acid oxidase test system was compared with that calculated on the basis that all the absorption at 450 $m\mu$ was due to flavin. The results of several experiments are shown in Table II. A maximum figure of 35% was obtained for the ratio

$$\frac{\text{flavin determined as flavindinucleotide}}{\text{flavin on basis of total absorption at } 450 \text{ } m\mu}$$

Table II

Stage	$\mu\text{g. apparent flavinphosphate/ml. as determined by height of } 450\text{ m}\mu\text{ band}$	$\mu\text{g. flavin-phosphate/ml. as determined in } d\text{-amino-acid test}$	$\frac{\text{actual flavin}}{\text{apparent flavin}} \times 100$
(i) After 1st $(\text{NH}_4)_2\text{SO}_4$ fractionation	0.53	0.14	26%
(ii) After alcohol precipitation	0.55	0.18	33%
(iii) After alumina adsorption	0.66	0.23	35%
(iv) After 2nd $(\text{NH}_4)_2\text{SO}_4$ fractionation	0.56	0.17	31%
(v) Final preparation	0.61	0.21	35%

By varying the conditions for liberating the flavin group the yields were for the most part lower but never higher than 35%.

The amount of flavin split off from the denatured flavoprotein can also be estimated spectrophotometrically. This requires rather more concentrated solutions, and again the conditions under which denaturation takes place determine the yield of flavin. Splitting in dilute HCl under the conditions described above gave the highest recovery of flavin. For example, 3 ml. of a solution of flavoprotein containing 51.6 $\mu\text{g.}$ apparent flavinphosphate per ml. were mixed with 1.2 ml. saturated $(\text{NH}_4)_2\text{SO}_4$ and 0.6 ml. N HCl. After 5 min. the precipitate was centrifuged off and the supernatant fluid neutralized with solid Na_2HPO_4 . The slight colourless precipitate which formed was centrifuged off and a clear yellow solution was thus obtained. After estimating spectrophotometrically the amount of flavinphosphate present from the height of the $450\text{ m}\mu$ band and allowing for dilution, the flavinphosphate content of the original solution was calculated to be 18.3 $\mu\text{g.}$ per ml. or 35% of that expected on the basis of the $450\text{ m}\mu$ absorption of the flavoprotein. Thus the two independent methods of estimating flavin are in good agreement that flavin accounts for about 35% of the total light absorption of flavoprotein at $450\text{ m}\mu$. On the other hand, 60% of the light absorption is abolished by addition of hydrosulphite indicating that another 25% of the total absorption, while not apparently due to flavin, is eliminated by reduction with hydrosulphite. In addition the hydrosulphite-reduced flavoprotein solution still contains some group absorbing 40% as much as the oxidized compound.

Previously Corran & Green [1938, 2] estimated that flavin accounted for 80% of the total light absorption of the flavoprotein at $450\text{ m}\mu$. This estimate has now been shown to be incorrect.

The available data allow the calculation of the approximate visible spectrum of the non-flavin group in milk flavoprotein (cf. Fig. 2). This was arrived at by subtracting the absorption of the flavin moiety from that of the flavoprotein. The band in the visible region shows a broad peak between 440 and 460 $\text{m}\mu$.

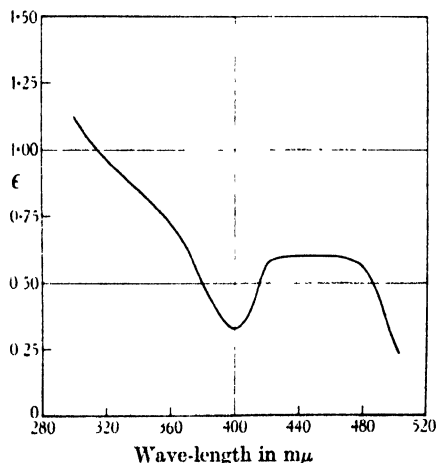


Fig. 2. Calculated absorption spectrum of non-flavin coloured group of milk flavoprotein (0.264%).

(4) *Properties of the flavin group*

We have used two methods for the isolation and study of the flavin group; the first involves splitting off the flavin from a highly purified sample of the flavoprotein; the second involves splitting the flavoprotein at a very crude stage. By the first method a few procedures suffice to reach the practically pure flavin but then the scarcity of starting material makes it difficult to obtain sufficient material for chemical investigations. The second method is more elaborate but it has the virtue of making the isolation less expensive.

(1) A sample of milk flavoprotein at the stage of extinction ratio 15 was heated at 70° for 5 min. in presence of 15% $(\text{NH}_4)_2\text{SO}_4$ and dilute acetic acid ($p\text{H}$ 3.8). After filtration the protein-free solution was saturated with $(\text{NH}_4)_2\text{SO}_4$ and extracted at *ca.* 70° with molten phenol. Water was added and the phenol was extracted with ether. The spectrum of the flavin thus obtained is shown in Fig. 3. A comparison of the relative heights of the three absorption bands at

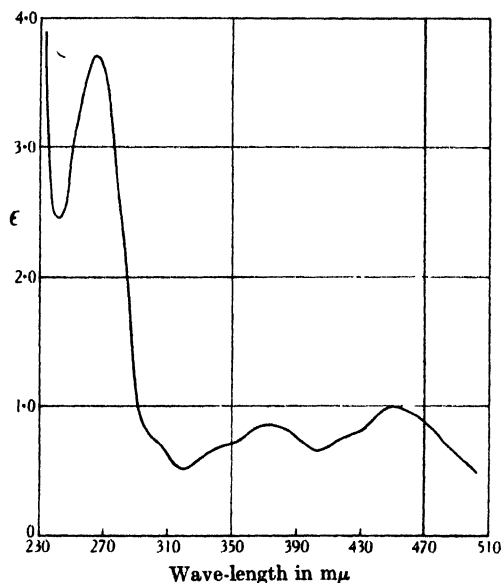


Fig. 3. Absorption spectrum of liberated flavin group of milk flavoprotein.

265, 370 and 450 $m\mu$ respectively with those for flavin adenine dinucleotide of yeast [Warburg & Christian, 1938] is given below.

λ in $m\mu$	Milk flavin	Yeast flavin
265	3.7	3.2
370	0.85	0.8
450	1.0	1.0

Ball [1939] found even closer agreement between the two respective spectra.

We have shown previously that the flavin can act as the coenzyme of the *d*-amino-acid oxidase. Comparison of the milk and yeast flavins shows that they have the same activity within the limits of experimental error.

$\mu\text{l. O}_2$ per 10 min. in test system	0.5 $\mu\text{g. flavinphosphate}$	
	Milk	Yeast
	68	76
	75	70

(2) Whey powder (cf. Corran & Green [1938, 2] for preparation) was shaken mechanically for 2 hr. with 75 % methanol. The insoluble protein was filtered off and the methanol removed by distillation *in vacuo*. The aqueous solution was saturated with $(\text{NH}_4)_2\text{SO}_4$ and heated to 80° . The heavy protein precipitate was filtered off. The filtrate was extracted with molten phenol at 70° . Water was added to the separated phenol phase and the phenol extracted with ether. The aqueous solution was acidified to pH 3 with nitric acid and a large excess of AgNO_3 added. The silver salt was decomposed with H_2S and the AgS filtered off. The flavin solution was then frozen and thawed. This procedure aggregated colloidal AgS which defied the first filtration.

The following are typical results carried out on the above flavin solution:

	Found	Theory for dinucleotide on the basis of flavin
Flavin	$0.82 \times 10^{-7} M$	—
Phosphorus	$1.78 \times 10^{-7} M$	$1.64 \times 10^{-7} M$
Nitrogen	$7.4 \times 10^{-7} M$	$7.4 \times 10^{-7} M$

Flavin was estimated spectrophotometrically assuming that the absorption coefficient $\beta = 2.6 \times 10^7 \text{ cm}^2$ per mole; phosphorus was estimated by the method of Kuttner & Lichtenstein [1932]. Miss V. Rogers kindly carried out the N estimations by the ultra-micro-Kjeldahl method of Needham & Boell [1939].

The orcin colorimetric method for the estimation of sugar [cf. Pirie, 1936] was used to demonstrate that carbohydrate was present in milk flavin in approximately the correct order of magnitude (80 %). Adenine was tested for as uric acid by conversion into hypoxanthine with nitrous acid, and then oxidation to uric acid by molecular oxygen in presence of the xanthine oxidase (unpublished method of Mr R. Markham). About 80 % of the theory for adenine was found.

Although the chemical analyses by themselves are not conclusive, yet, taken in conjunction with the close similarity of milk and yeast flavins with respect to spectrum and catalytic activity they leave little doubt of the identity of the two compounds.

Various attempts were made to split off flavin reversibly from the flavo-protein. Dialysis of the flavoprotein solution for long periods against dilute acetic acid and dilute HCl at 0° led to the removal of the flavin without apparent denaturation of the protein. But it was not found possible to produce an active enzyme by adding milk dinucleotide to the flavin-free protein. Negative results were also obtained by the application at low temperatures of the $\text{HCl}-(\text{NH}_4)_2\text{SO}_4$ method of Warburg & Christian [1938] used with such striking success in the splitting of the *d*-amino-acid oxidase and yeast flavoprotein. Ball [1939] has succeeded in the reversible resolution of milk flavoprotein by prolonged dialysis against distilled water at 0° . This method has failed in our hands. In fact we have consistently used the dialysis procedure as a successful means of purifying milk flavoprotein in the final stages.

(5) Catalytic properties

Milk flavoprotein catalyses the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I. A summary of the catalytic constants at the stage of extinction ratio 6.2 is given in Table III. The three catalytic activities are of the same order of magnitude. The concentrations of hypoxanthine and aldehyde used were sufficient to saturate the enzyme, thus ensuring maximal activity in each case. The problem of saturating the enzyme in respect to dihydrocoenzyme I is somewhat complicated and the reader is referred to Corran & Green [1938, 2] for the conditions under which maximal activity is reached. Crude preparations of the

Table III. *Summary of catalytic properties*

	Hypoxanthine	Acetaldehyde	Dihydrocoenzyme I
Methylene blue reduction time	1.12 min. ⁽¹⁾	0.60 min. ⁽²⁾	1.33 min. ⁽³⁾
Relative activities (hypoxanthine = 100)	100	186	85
Turnover number	306	570	260
Q_{MB} * per mg. flavinphosphate	9×10^4	1.67×10^4	7.6×10^4
Q_{MB} per mg. protein	2420	3500	2060

(1) 1 ml. buffer (7.2), 0.1 ml. 0.0113*M* methylene blue, 0.2 ml. 0.5% hypoxanthine and 0.3 ml. of a flavoprotein containing 5.35 μ g. actual flavinphosphate/ml. (15.3 μ g. apparent flavinphosphate).

(2) Details as for (1) except 0.2 ml. *M* acetaldehyde in place of hypoxanthine.

(3) 1 ml. lactic enzyme, 1 ml. 2% coenzyme I, 0.2 ml. 2*M* HCN, 0.1 ml. 0.0113*M* methylene blue, 0.2 ml. *M* lactate and 0.3 ml. of flavoprotein solution as in (1).

* $Q_{MB} = \mu$ l H_2 transferred to methylene blue per hr. per mg. dry weight.

xanthine oxidase in presence of hypoxanthine react at the same speed with methylene blue as with O_2 [Green & Dixon, 1934]. The Q_{MB} (μ l H_2 transferred to methylene blue per hour per mg. dry weight) can therefore be considered to be roughly equivalent to the Q_{O_2} . The difficulties of making aerobic measurements with highly purified flavoprotein preparations were explained in Section 1.

(6) *The association of the three catalytic activities with the flavoprotein*

All the available evidence has been consistent with the view that our milk preparations contain only one flavoprotein at all stages of purification. The possibility, however, cannot be excluded that our flavoprotein preparations are mixtures of two or three flavoproteins of similar molecular weight, spectrum and chemical properties and that this mixture cannot be resolved by the procedures used. The possibility is indeed remote but it cannot be dismissed until more precise and varied criteria of the homogeneity of proteins become available. For ease of presentation, however, we shall assume that we are dealing with one flavoprotein.

Table IV. *Proportionality between hypoxanthine activity and apparent flavinphosphate content at different stages of purity*

Stage	1 Hypoxanthine activity (units/ml.)	2 Apparent flavin- phosphate (μ g./ml.)	2/1
1st $(NH_4)_2SO_4$ fractionation	5.2	26.4	5.1
After alcohol precipitation	5.8	27.4	4.7
After 1st alumina adsorption	4.0	16.4	4.1
After 2nd alumina adsorption	3.2	14.2	4.4
Ratio 8.3	4.3	22	5.1
Ratio 7.4	8.8	41.6	4.7
Ratio 6.4	5.0	22	4.4
Ratio 6.2	3.3	15.3	4.6
		Average	4.6

Table IV shows the proportionality between the enzyme units (hypoxanthine) and the apparent flavinphosphate content of various flavoprotein preparations. A strict proportionality exists at all stages of purification. If the three activities are associated with the same flavoprotein we should expect the activity ratio, hypoxanthine : aldehyde : dihydrocoenzyme I to be identical at all stages. Such has been found to be the case. Dixon [1939] and Booth [1935] have already established that xanthine oxidase of milk is a xanthine-aldehyde oxidase. Ball

[1939] in turn has identified the xanthine-aldehyde oxidase with milk flavoprotein. Finally our results identify the xanthine-aldehyde and the dihydrocoenzyme oxidase with the same flavoprotein.

The three catalytic activities, although apparently associated with the same flavoprotein, are not invariant functions of the flavoprotein. Preparations which have been dried or treated with cyanide lose activity towards hypoxanthine and aldehyde but are still active towards dihydrocoenzyme I. It is significant that hypoxanthine and aldehyde activities always run parallel. No procedure has yet been devised which will eliminate dihydrocoenzyme I activity without affecting either xanthine or aldehyde activity. The hypoxanthine-aldehyde function is fragile compared with that of dihydrocoenzyme, and the two functions clearly must involve different active groups in the protein part of the molecule.

Inactivation by drying. A sample of milk flavoprotein at the stage of extinction ratio 7.0 was frozen and dried in high vacuum over H_2SO_4 . Tests of the redissolved flavoprotein showed that 75 % of the activity towards hypoxanthine was lost. No apparent difference was observed in the spectrum of the flavoprotein or in its solubility. The inactivation was not, therefore, the result of any gross alteration in the flavoprotein molecule.

At lower stages of purity, inactivation was a slower process. For example, at the stage of extinction ratio 50, drying for 3 days under the above conditions brought about only a 50 % loss in hypoxanthine activity although dihydrocoenzyme I activity was unaffected. Dried whey powder retained xanthine-aldehyde activity for a period of months. But here again deterioration was taking place though at a slower rate. For example, a whey powder which had stood for 4 months yielded a flavoprotein which was completely inactive towards hypoxanthine and aldehyde though still active towards dihydrocoenzyme I. This inactivation by drying took place equally well in presence or in absence of salts.

We have repeated the isolation of milk flavoprotein by the original method of Corran & Green [1938, 2] with a view to determining the exact stages at which xanthine-aldehyde activity was lost. The following results were obtained:

Stage	Enzyme units (hypoxanthine)
1st dried preparation (whey powder)	620
2nd dried preparation	90
After lead treatment	0

There was about a 30 % loss of flavoprotein in proceeding from the first to the second dried preparations whereas the loss in hypoxanthine activity was 85 %. After the lead treatment the flavoprotein present was completely inactive towards hypoxanthine.

Inactivation by cyanide. Dixon & Keilin [1936] have shown that xanthine oxidase when incubated with cyanide becomes irreversibly inactivated. We have utilized this effect as a tool for the rapid elimination of xanthine-aldehyde activity without affecting dihydrocoenzyme I activity.

A preparation of the flavoprotein at the stage of extinction ratio 15 was made $M/10$ with respect to HCN . After 10 min. the flavoprotein was precipitated with half-saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was washed with a large volume of half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution and then dissolved in phosphate buffer. The ratio, hypoxanthine activity: dihydrocoenzyme I activity was found to be 1.0 : 2.6 instead of the usual ratio of 1.0 : 0.85. The above procedure was then repeated. After this treatment no more hypoxanthine-aldehyde activity was present although dihydrocoenzyme I activity was unimpaired and the spectrum and chemical properties of the flavoprotein were not noticeably altered.

(7) *Mechanism of reaction*

The flavoproteins hitherto described in the literature are known to catalyse the oxidation of their respective substrates by undergoing a cycle of reduction by the substrate and then oxidation by O_2 or some other oxidizing agent. This cycle concerns only the flavin part of the molecule and hence flavin is regarded as the prosthetic group. By analogy we should expect a similar mechanism to apply to milk flavoprotein. The analogy however is not complete. Whereas the other flavoproteins contain only one coloured prosthetic group, viz. flavin, milk flavoprotein contains some other coloured grouping in addition to flavin which may be concerned in the catalytic role of the enzyme. Furthermore, milk flavoprotein contains 2-3 mol. of flavin per mol. of protein whereas the ratio is 1:1 for the other flavoproteins. Finally, milk flavoprotein catalyses three distinct oxidations in contrast to the one specific reaction catalysed by the other flavoproteins. These differences in properties do not exclude a mechanism involving cyclical reduction and oxidation of the flavin groups but they weaken the force of analogy and call for caution in the interpretation of results.

A strong solution of milk flavoprotein is orange-red in colour. On addition of hydrosulphite the colour is practically bleached except for a residual pale brown colour. It is interesting to note that although to the eye the colour has largely disappeared yet spectrophotometric estimation discloses 40% of the original light absorption at $450\text{ m}\mu$. On addition of hypoxanthine or aldehyde to the flavoprotein under anaerobic conditions this colour change does not take place at once. After incubation at 38° for 15 min. there is a slight but definite diminution of colour compared with the untreated control. The paling becomes more accentuated with time. After some hours' incubation the flavoprotein solution takes on the appearance of the hydrosulphite-reduced control. Admission of air restores the original colour in the tubes containing either hypoxanthine or hydrosulphite as reductant for the flavoprotein. These experiments show that the flavin groups are slowly reduced by hypoxanthine and aldehyde, and that reduced flavin is autoxidizable in air. The rate, however, is some thousand times slower than would be expected if the flavin groups were reduced and oxidized in the course of catalytic activity. The following experiment clearly demonstrated this discrepancy in velocity: 1 ml. of a solution of the flavoprotein containing $35\text{ }\mu\text{g}$. apparent flavinphosphate per mol., i.e. $12\text{ }\mu\text{g}$. actual flavinphosphate was mixed with 1 mg. hypoxanthine under anaerobic conditions. About 2 hr. were required before the complete bleaching of the flavin colour was pronounced. A parallel test showed that 1 ml. of the flavoprotein solution in presence of hypoxanthine catalysed the reduction of 0.1 ml. of $0.0113M$ methylene blue in 7 sec. Assuming that the reduction of methylene blue by hypoxanthine involves a cycle of the flavin groups the ratio ($\mu\text{l. H}_2$ transferred to methylene blue : $\mu\text{l. H}_2$ equivalent of flavoprotein) should give the number of times each flavin group would have to be reduced and oxidized in 1 min. The ratio turns out to be 306. It follows, therefore, that under anaerobic conditions the flavin groups should have been reduced in *ca.* 0.2 sec. The observed time was more like 2 hr.

The possibility arises that the substrate reduces a small part of the flavin groups at once and that the slow reduction of the bulk of the flavin groups has nothing to do with the catalysis. To test this hypothesis it would be necessary to make rapid spectrophotometric measurements of the flavoprotein immediately after mixing with the substrate under anaerobic conditions. Unfortunately we have been unable to carry out such an experiment. Measurements made within 10 min. of mixing invariably show partial reduction of the flavo-

protein though the degree of reduction observed varies from one preparation to another.

To summarize we may say that none of the three substrates can rapidly bleach the flavoprotein to completion. No decision has been reached as to whether there is an instantaneous partial reduction of the flavoprotein by the three substrates. Furthermore, there does not appear to be summation of the substrates with respect either to the degree or velocity of reduction. The interpretation of these results must await more knowledge of the nature of the additional coloured grouping in the molecule. The hypothesis of a flavin cycle is neither excluded nor supported by our experiments.

Ball [1939] has reported that both hypoxanthine and aldehyde reduce milk flavoprotein rapidly and almost as completely as hydrosulphite. This observation is difficult to explain except on the basis that the flavoprotein isolated by our method is a mixture of flavoproteins only a small proportion of which is represented by the xanthine-oxidase flavoprotein whereas that of Ball's consists principally or entirely of xanthine-oxidase flavoprotein. However, comparison of the catalytic activities of the flavoproteins isolated by Ball's and our methods does not support this possibility.

(8) *Liver xanthine oxidase*

The isolation of the xanthine oxidase offered an independent method of confirming the conclusions reached with the milk enzyme. We have succeeded in purifying the liver enzyme to a point where the flavoprotein colour in the preparation can be seen directly, but as yet coloured impurities have not been sufficiently removed to permit satisfactory spectroscopic measurements.

The following is a brief résumé of the method of isolation. Minced pig liver was mixed with 3 vol. water and 0.4 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 4.6. The insoluble material was discarded by centrifuging; the supernatant fluid was brought to 60% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitate was filtered and resuspended in water—insoluble material being discarded by centrifuging. The dialysed solution was acidified to pH 4.6 at 0° and in presence of 16% alcohol. The precipitate was dissolved in buffer at pH 7.2 and some inactive protein removed by heat-coagulation at 56°. The solution was finally fractionated between the limits of 45 and 60% saturation of $(\text{NH}_4)_2\text{SO}_4$.

The final solution was strong yellow in colour. Only part of the colour was bleached by hydrosulphite. Shaking with air restored the original depth of colour. The presence of a flavoprotein in the preparation containing bound flavin adenine dinucleotide was also demonstrated by splitting off the flavin group and observing a significant O_2 uptake in the *d*-amino-acid oxidase test system.

The above preparation of liver flavoprotein catalysed the oxidation of hypoxanthine, aldehyde and dihydrocoenzyme I:

	Relative activities	
	Liver	Milk
Hypoxanthine	100	100
Aldehyde	28	186
Dihydrocoenzyme I	56	85

The ratio of the three activities was not the same as for milk. Aldehyde had 0.28 the activity of hypoxanthine in the case of the liver enzyme and 1.86 the activity of hypoxanthine in the case of the milk enzyme. The significant fact, however, is that all three catalytic activities are associated with both the liver and the milk flavoproteins.

The highest Q_{MB} reached with the xanthine oxidase of liver was 400 as compared with 2400 for the best preparation of milk enzyme. The degree of concentration relative to the original liver was *ca.* 200. Work on the purification is still in progress.

Pig liver contains an aldehyde oxidase in addition to the hypoxanthine-aldehyde oxidase. This enzyme, however, was removed early in the purification and could not account for the aldehyde activity observed in the purified flavoprotein preparation.

The xanthine oxidase of liver was extremely unstable at all stages of purification. Only by rapid manipulation was it possible to reach the higher stages of purity without excessive loss in activity.

SUMMARY

A method is described for preparing a flavoprotein which catalyses the oxidation of hypoxanthine, aldehydes and dihydrocoenzyme I, and is *ca.* 1000 times more active per mg. dry weight than milk. The three catalytic activities although associated with the same flavoprotein can be differentially inactivated. Drying and incubation with cyanide abolish hypoxanthine-aldehyde activity without affecting either dihydrocoenzyme I activity or the spectrum and chemical properties of the flavoprotein.

The flavin moiety has been shown to be very similar to if not identical with flavinadenine dinucleotide. Flavin accounts for only 35 % of the total absorption at 450 m μ . Evidence is presented for the existence of a non-flavin coloured group in the molecule. The catalytic role of this additional group has yet to be clarified. No direct evidence has been obtained that the flavin groups undergo a cycle first of reduction by the three substrates and then of oxidation by some oxidizing agents.

We are grateful to Mr S. Williamson and Mr B. Slater for their valuable assistance. The research has been made possible by grants from the Medical Research Council and the Royal Society.

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ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

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(with technical assistance by E. DODWELL)

The new specimen of milk flavoprotein described above was ultracentrifuged at 54,000 r.p.m. in a 12 mm. cell at a concentration of approximately 0.5% (as judged from the refractive increment 0.00097).

Fig. 4 shows a photograph by the "diagonal schlieren" method. Three components are present, with $S_{20} \times 10^{13}$ equal to 6.8, 12.6 and 19.2. Comparison with the previous results [Philpot, 1938, 1] shows that the first two components correspond with the ones formerly labelled " γ " and " δ ", while the third component " ϵ " is a new one peculiar to the latest specimen. The " δ " component, which is the flavoprotein itself, is now for the first time the most prominent. Integration shows that it accounts for 68% of the total refractive increment, or 81% of the observably sedimenting material. The " γ " and " ϵ " components account for 11 and 5%, respectively, of the total refractive increment, while the remaining 16% is due to uncentrifugable or highly polydisperse material. These figures are all \pm about 5%.

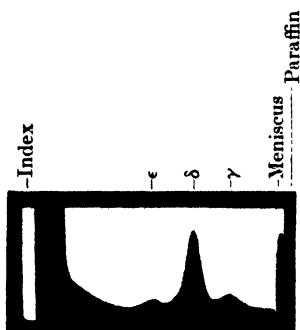


Fig. 4.

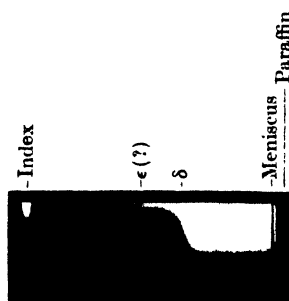


Fig. 5.

Fig. 5 shows a photograph taken during the same run by a self-plotting absorption method. The optical system is identical with that of the diagonal schlieren method [Philpot, 1938, 2]; but the diagonal edge is set vertical, to let all the light through, and an Ilford carbon-gelatin wedge, with horizontal gradient, is placed in front of the photographic plate. With monochromatic light of suitable wave-length any line of constant intensity plots concentration against distance from the meniscus. With imperfectly monochromatic light a wedge cell containing the protein is preferable to the carbon wedge, but this requires a lot of material.

The prominent S-shaped step in Fig. 5 shows that most of the light absorption at 475 $m\mu$, the wave-length used, is due to a single component, whose sedimentation constant is 12.6×10^{-13} , and which is therefore the " δ " component. On close inspection it is possible to see that 5–10% of the light absorption is caused by faster-moving material with sedimentation constant about 17×10^{-13} . This

may mean that the "ε" component of Fig. 4 is coloured; but the accuracy of the absorption method is insufficient to give any certainty. If the "ε" component were coloured it could be a twofold aggregate of the main "δ" component. An attempt to dissociate it with 0.01*M* hexametaphosphate was, however, unsuccessful. None of the coloured material is uncentrifugable.

If the "δ" component forms 60–80 % of the material and contains 90–100 % of the flavin, if the overall percentage of flavinphosphate is 0.27 % as found above, and if the molecular weight is 220,000–320,000, then there must be 1.4–3.1 flavin groups per molecule. The previous conclusion that there were eight groups per molecule was based on a higher flavin percentage which has since been revised.

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CCXII. THE PROPERTIES OF ANTI-GENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*

IV. THE HYDROLYSIS OF THE FORMAMINO LINKAGE

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THE predominating antigenic substance present in smooth strains of *Br. melitensis* has been isolated and described in some detail; it appears to be a complex of some phospholipins, a protein-like substance and the formyl derivative of a polyhydroxy amine [Miles & Pirie, 1939, 1, 2]. This complex "native" antigen may be successively deprived of the phospholipins and the protein-like substance. The formamino compound which remains is apparently fully antigenic and retains the serological activity of the native antigen. At this level of complexity the antigen was for convenience called AP. After precipitation with ammonium sulphate it has mol. wt. about 1,000,000 which may be reversibly reduced to 100,000–200,000 by disintegration with sodium dodecyl sulphate. Hydrolysis of AP in *N*/10 HCl for 5–10 min. at 100° liberates another phospholipin (PL 2) and partially liberates an inorganic phosphate; in 45–60 min. the phospholipin is coagulated and the phosphate fully liberated [Miles & Pirie, 1939, 3]. After this much hydrolysis the formamino compound is still retained by a cellophane membrane, but it will no longer precipitate with homologous antisera.

Many modifications of the method of preparing this phospholipin-free formamino compound have been tried. The method already published seems to be the most convenient although there is some liberation of amino groups during the hydrolysis. The resulting mixture of formylated amine and partly deformylated material can be fractionated to some extent either by dialysis or by precipitation with alcohol in the presence of salts. After these treatments the less readily diffusible material and the more readily precipitable materials have been the best preparations obtainable. By drying frozen solutions the formylated amine is obtained as a fluffy product which is readily soluble in water, and which should contain less than 0.6% amino-N (Van Slyke). AP, the complex of formylated amine and phospholipin, on the other hand, dissolves in water with difficulty if it has once been dried, and contains no detectable amino-N. In this paper the further stages in the hydrolysis of the formylated amine will be described.

Formic acid has been identified as a product of the hydrolysis of AP by a number of different methods and we have produced evidence that it is linked to an amino group. The question has been studied in some detail for formyl derivatives have not, apparently, been described before in nature if we exclude substances such as chlorophyll *b* [Fischer & Breitner, 1935] and *Spirographis haemin*

[Fischer & von Seemann, 1936] which, although described as formyl derivatives, are simply aldehydes. Formic acid is, of course, well known in the free state, e.g. in ants and in fruit juices, and in small quantities it has often been recognized in the products of vigorous hydrolysis of organic materials by acids and alkalis [e.g. Sundwik, 1881]. Adenine [Stevens, 1937] and lycoperdin [Kotake & Sera, 1913] are peculiar in giving high yields of formic acid on hydrolysis but only under conditions of a different order of intensity from those used with the *Br. melitensis* amine. Lycoperdin, which is isolated from the acid hydrolysate of puff balls, on hydrolysis yields glucosamine as well as formic acid, but the amino groups appear to be freed at a level of hydrolysis below that required to liberate the formic acid.

The fact that formyl groups have not been found more often in nature cannot be taken as evidence that they are in fact rare, for the identification of volatile acids is often not very conscientiously carried out. It is probable that some of the "acetyl" groups that have been described are in fact formyl groups.

Identification of formic acid

Quantitative estimation of the volatile acid liberated on hydrolysis can be carried out by the method of Elek & Harte [1936]; by this method 1 equiv. of acid is given by 270–300 g. of AP and, if this is reckoned as formic acid, it corresponds to 9.5–11 % of formyl groups. In this estimation *p*-toluenesulphonic acid is used for the hydrolysis, but if one wishes to work up both the base that remains in the distillation flask and the volatile acid, sulphuric acid has obvious advantages, although with this acid recovery cannot be quantitative since the mixture cannot be distilled to dryness. 2 ml. 2*N* H₂SO₄ were used for the hydrolysis of 100–700 mg. quantities of AP or formylated amine. After refluxing for 30 min. the mixture was allowed to distil with the addition of water to keep the volume constant. About half the volatile acid comes over in 30 ml. of distillate and there is some charring of the materials in the distillation flask. The distillate was neutralized with 0.36*N* Ba(OH₂), concentrated to 2 ml., centrifuged from a trace of solid and allowed to crystallize in a desiccator. It was clear, from microscopical examination, that this product was slightly contaminated with gum. The latter could be removed by recrystallization from water or, more conveniently, from dilute alcohol. More crude Ba salt could be isolated from the mother liquors making the total yield 75 % of the original weight of the salt; it is clear therefore that this salt is the principal component of the neutralized distillate. The gummy residue still contained about 50 % of barium formate, as estimated by the mercuric chloride technique; its other constituents have a much higher carbon content but they have not been studied further.

Several samples of Ba salt, recrystallized from water, have given the following analyses (Weiler): C, 11.32; H, 1.08; Ba, 60.3 %. The C and H figures are appreciably higher than the theoretical (C, 10.6; H, 0.8; Ba, 60.2 %), but a sample of barium formate, prepared from Kahlbaum's formic acid and analysed by the same analyst at the same time gave similar results. Furthermore, the figures differ widely from those for any other fatty acids; the other acids which would give Ba salts with such high Ba and low C contents are non-volatile or give Ba salts that are not soluble in water at neutrality.

The formic acid which remains in the flask after distillation can be recovered by extracting the acid fluid several times with methyl ethyl ketone for, as Archibald [1932] has shown, the partition coefficient is 1.3 and is, therefore, greater with this solvent than with the others that have been tested. Barium formate is prepared from this extract by counter-extraction with enough aqueous

$\text{Ba}(\text{OH})_2$ to make the mixture alkaline; the aqueous layer is worked up as before. This fraction is microscopically purer than that made from the distillate but, after recrystallization, it gives the same analytical figures. The total yield of barium formate from these two fractions is 70 % of what would be expected from the N content of the starting material, e.g. 164 mg. of formylated amine containing 6.5 % N gave 61 mg. of barium formate. In some other experiments the distillates were neutralized, or the methyl ethyl ketone extracts counter-extracted, with aqueous NaOH and dried. Lead formate was prepared from the crude sodium formate by adding the corresponding amount of lead nitrate solution and evaporating if necessary. Lead formate is sparingly soluble and crystallizes well and is therefore convenient for the characterization of the acid, but the isolation of lead formate cannot easily be combined with a study of the other components of the distillate or extract. After recrystallization it gave the following analytical figures: found: C, 8.3; H, 0.87 %. Calculated: C, 8.08; H, 0.674 %.

Distillates from many hydrolysed bacterial fractions have been tested for the presence of acetic acid by the colour reaction with lanthanum salts and iodine [Kruger & Tschirch, 1930]. Formic acid gives a yellowish opalescence with this reagent but the blue colour due to acetic acid can readily be detected in its presence. Acetic acid has never been detected in preparations which had not been artificially acetylated and we have never had reason to suspect the presence of large amounts of any other volatile acid. Pennell & Huddleson [1937] found a fraction in *Br. melitensis* which, after saponification, gave a yield of volatile acid corresponding to an acetyl content of 48 %; we have never encountered such a fraction.

The evolution of CO on incubation with H_2SO_4 is a characteristic property of formic acid and the formates. The action proceeds quickly at 37° and may conveniently be followed in the Warburg manometer. 3 ml. of conc. H_2SO_4 are put in the main chamber of the manometer and dry barium formate in the side bulb; after equilibration the two are mixed.

In one experiment with 1.04 mg. of barium formate the pressure rose to 142 mm. of Brodie's fluid in 10 min., to 159 mm. in 20 min. and to 160 mm. in 25 min. The manometer used had a volume of 16.9 ml. Since the correction for the solubility of CO in H_2SO_4 is small it is legitimate to use the value 0.024 which Christoff [1906] found for the solubility coefficient in 95.6 % H_2SO_4 . The reaction was carried out at 37° and 764 mm.; the weight of CO liberated is therefore

$$\frac{28 \times 160 (13.9 + 0.024 \times 3) \times 273 \times 760}{100,000 \times 22.4 \times 310 \times 764} = 0.251 \text{ mg.}$$

This is 102 % of the theoretical value. By the same technique commercial samples of barium formate have given 95–105 % of the expected pressure rise.

Formic acid is often estimated by titration with potassium permanganate. Barium formate made from the antigen gives satisfactory results which will not be recorded since the method has little specificity.

The formamino linkage

In the course of determining the mode of linkage of formic acid it was necessary to compare the rates of production of formic acid and of amino groups during hydrolysis; for this purpose the acid distillation method is clearly unsuitable but a method based on the extraction of the acid with methyl ethyl ketone was convenient. Small amounts of methyl ethyl ketone do not interfere with the standard and apparently specific method of estimation based on the reduction of mercuric to mercurous chloride; 1 ml. quantities of a 1.0 % solution

of formylated amine in 0.33*N* HCl were withdrawn after various periods of heating in a boiling water bath and shaken vigorously with two 5 ml. quantities of methyl ethyl ketone. The combined extracts were mixed with 0.5 ml. of 0.5*N* Na₂CO₃, evaporated to dryness on a water bath and neutralized or slightly acidified with acetic acid. 10 ml. of a saturated solution of mercuric chloride containing 2% of acetic acid were added and the mixture heated in a stoppered 50 ml. flask in a boiling water bath for 2 hr. The calomel was filtered off on a small, weighed, sintered glass funnel, washed with *N* HCl, water and alcohol and weighed after drying at 100°. Control experiments with known quantities of formic acid showed that the reduction proceeded quantitatively with 0.5–2.0 mg. of acid and that formic acid was 75–80% extracted by the two treatments with methyl ethyl ketone. In Table I the results of such an experiment are recorded and also the

Table I

1 Time of heating min.	2 Amino-N %	3 Formic acid found i.e. wt. of Hg ₂ Cl ₂ × 10 ⁻³ %	4 Formic acid found expressed as % of the amount of formic acid corresponding to the amino-N in col. 2
0	0.8	0	—
10	2.8	6.7	73
15	3.7	9.6	79
40	4.8	13.2	83
120	6.6	16.4	76

The recovery of formic acid by the method used is 75–80%.

amino-acid content of the fluid after various periods of hydrolysis. It can be seen that the formic acid and amino groups are liberated at similar rates and it is, therefore, reasonable to assume that the starting material was an *N*-formyl derivative. Experiments of this type cannot be carried out satisfactorily with unhydrolysed AP, for the PL2 that is liberated during the hydrolysis interferes with the separation of the two liquid layers.

The deformed compound

Solutions of AP from which the phospholipin components, the phosphate and the formyl group have been removed react with nitrous acid under the same conditions as α -amino-acids and glucosamine. Using the usual Van Slyke technique a volume of gas is evolved which corresponds to 90% of the total N (Dumas). This agreement is satisfactory but, although glucosamine itself behaves like an amino-acid under these conditions, other amino-sugars and related substances have been found to give anomalous results [Levene & Meyer, 1923]. The amine, but not the formylated substance, also gives a "ninhydrin" reaction as strong as that given by the same weight of glucosamic acid or leucine and for this reason it was at first thought that the hydrolysate contained an amino-acid. That this is not the case is clear for two reasons: no CO₂ is evolved when the material is boiled with ninhydrin under the conditions used by Van Slyke & Dillon [1938] although a deep blue precipitate is formed; also, there is in the hydrolysate no titratable group with a *pK* in the usual range for carboxyl groups, i.e. between 5 and 2.

Titration curves have been plotted, using a double hydrogen electrode system [cf. Pirie & Pinhey, 1929], for AP, formylated amine and free amine. In an earlier paper [Miles & Pirie, 1939, 1] we stated that AP, which had been precipitated by alcohol from acid solution and then washed thoroughly with alcohol, behaved like

an acid with equiv. wt. 8000. This has been confirmed by comparing the titration curve of a 1.5% solution of AP with that for the same volume of water. Maximum buffering occurs between pH 3 and 4. As hydrolysis proceeds a new group with pK at 6.8 appears and the amount of acid used in titrating this group is roughly proportional to the amino-N content of the preparation, e.g. a sample of formylated amine with 0.6%, or less, amino-N had equiv. wt. 2000 and one with 1.3% had equiv. wt. 950, whereas preparations from which the formic acid had been completely hydrolysed had about 7% amino-N and equiv. wt. 186. Although it is clear that glucosamine itself is not a constituent of AP, its titration curve was plotted for comparison since, so far as we are aware, the dissociation constants of the amino-sugars have not hitherto been measured. The observed value for pK_a at 19° for a 0.033*M* solution in the presence of 0.037*M* NaCl was 7.8. This value suggests that glucosamine might be useful as a buffer in the neutral range with certain systems.

Comparison of the amine and its formylated derivative

Alcohol will precipitate AP from solution and, with more difficulty, the formylated amine also. The free amine in neutral or alkaline solution is not easily precipitated, but its salts, especially the sulphate, separate as heavy oily layers when alcohol is added in small quantities to their strong aqueous solutions. Other solvents, such as methyl ethyl ketone, have the same effect. The separation of this oil causes difficulty in the removal of formic acid from hydrolysates unless dilute solutions are used. With other precipitants there are similar differences between the free amine and its formyl derivative. Phosphotungstic acid will only precipitate AP and the formylated amine completely from strongly acid solutions (e.g. 0.5*N* H₂SO₄) and the precipitates dissolve easily in water when washed. The free amine, on the other hand, can be precipitated from only slightly acid solution and the precipitate can be washed with water. For complete precipitation the amine requires about six times its weight of phosphotungstic acid whereas the other materials are precipitated by half this quantity. It is interesting to note that lycoperdin which, as has been stated, yields glucosamine on hydrolysis, is also precipitated by phosphotungstic acid although glucosamine itself is not. Picric, picrolonic and flavianic acids precipitate aqueous solutions of the amine but do not precipitate the formylated amine or AP. These precipitates are all gummy and amorphous although the third gives a birefringent gum which can be orientated by shearing. Attempts have been made to get crystalline salts with a large number of acids but so far they have been unsuccessful.

The presence of material with a high C content in the crude preparations of barium formate, made by neutralizing the distillates from hydrolysates, has already been mentioned. A similar material is left when the methyl ethyl ketone solution that remains after counter-extraction is evaporated. These substances account for less than 5% of the formylated amine, but we have no evidence that the yield is constant or that they are primary hydrolysis products of the molecule. It is probable, however, that they are formed or liberated during the hydrolysis, for they dissolve in the ordinary organic solvents whereas nothing can be extracted from AP or formylated amine by those solvents.

Solutions of the amine and its formyl derivatives are not precipitated by homologous *Brucella* antisera. Nevertheless, the various fractions isolated from hydrolysates of AP may be compared serologically by measuring their capacity to inhibit agglutination of *Br. melitensis* by a monospecific serum. The minimum concentrations producing a standard degree of inhibition are listed in Table II.

Table II. *Specific inhibiting titres*

Starting material	Nature of solution tested	Inhibition titres (expressed as conc. of starting material)
1. AP (gentle hydrolysis)	(a) AP	1:70,000
	(b) AP partly split into phospholipin and formylated amine	1:40,000
	(c) AP completely split into phospholipin and formylated amine	1:12,000
2. Dialysed preparation of formylated amine	(a) Formylated amine	1:12,000
	(b) Amine	1:250
	(c) Partly hydrolysed amine	>1:50

The inhibition titre of AP in Table II is about four times greater than that already recorded for AP [Miles & Pirie, 1939, 2], for, although the same method of titration has been used, the end-points are in this case taken as the concentration reducing the degree of agglutination by 50 %. This end-point can be determined with greater accuracy than the one previously measured, namely, the concentration producing total inhibition.

The inhibiting power of AP appears to depend in part on the integrity of the complex of formylated amine with the phospholipin PL2. Complete conversion of AP into a suspension of phospholipin in a solution of the amine reduced the titre to 1:12,000. If this residual inhibition is due to the free formylated amine in the mixture, which contains about 20 % by weight of the phospholipin, the inhibiting titre of the formylated amine alone should be in the region of 1:15,000. The observed figure of 1:12,000 (Table II, 2 (a)) is sufficiently close to warrant this assumption. Deformylation results in a big reduction of inhibiting power, and hydrolysed amine is inactive in concentrations as large as 2 %. Inhibiting power is reduced progressively with the loss of phospholipin or formyl groups and with destruction of the amine. Though it is likely that these three chemical structures are the important antigenic determinants of AP, it is impossible on the available evidence to exclude the existence of an unrecognized constituent which is progressively liberated in the course of a hydrolysis whose stages are independently defined by the loss of phospholipin and the formyl groups.

In the following paper some preliminary conclusions on the constitution of the high-molecular amine which results from this hydrolysis will be presented.

SUMMARY

Formic acid has been identified as a product of the hydrolysis of the antigen of *Brucella melitensis* and evidence is presented that the unhydrolysed antigen contains a formamino group.

Some chemical, physical and serological properties of the high-molecular amine are compared with those of its formyl derivative.

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CCXIII. THE PROPERTIES OF ANTI-GENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*

V. HYDROLYSIS AND ACETYLATION OF THE AMINO-POLYHYDROXY COMPOUND DERIVED FROM THE ANTIGEN

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With an Addendum by J. ST L. PHILPOT

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IN the preceding papers [Miles & Pirie, 1939, 3, 4] stages in the hydrolysis of the antigen of *Brucella melitensis* were described, the final product being a complex amine. This amine is prepared from its formyl derivative by hydrolysis. Considerable discoloration occurs when hydrochloric or sulphuric acids are used for the hydrolysis; if nitric acid is used there is no discoloration but a partly deaminated product results. The free amine can be obtained nearly colourless by refluxing a 2% solution of AP, or better of the formylated amine (i.e. AP from which the phospholipin has been removed by very gentle acid hydrolysis), for 1 hr. with $N H_2SO_4$ and fractional precipitation with phosphotungstic acid. The first fifth of the phosphotungstic acid needed for complete precipitation brings down nearly all the colour; this precipitate is centrifuged off and the precipitation is then completed. The two precipitates are decomposed separately with baryta in the usual way and the excess of barium is removed exactly with sulphuric acid. The solution dries to a colourless scaly material.

It was stated previously [Miles & Pirie, 1939, 1] that AP contained 5.4% N and 0.1–0.2% S; recently, by working up preparations of AP that had been disintegrated with sodium dodecyl sulphate [Miles & Pirie, 1939, 2] preparations have been made that contain less than 0.1% S and 6.0–6.2% N. The amine that is derived from native AP contains less than 0.1% S and less than 0.05% P, for all the P in AP has been eliminated as phosphate and phospholipin. The other analytical figures were somewhat variable but fell in the following ranges: C 43–47; H 6.0–8.4; N 7.5–8.3%.

AP has $[\alpha]_D^{20} + 43^\circ$; the free amine has $[\alpha]_D^{20} + 72^\circ$ at about pH 9 and $+ 61^\circ$ when dissolved in $N/15 H_2SO_4$. This increase in specific rotation is of the order that would be expected on the hypothesis that the 25% of phospholipin (PL 2) in AP contributes nothing to the rotation and that the loss of this and of the formic acid does not affect the asymmetric structures in the molecule.

In the Addendum to this paper Philpot describes the ultracentrifuging of both the amine and its formyl derivative. If we make the usual assumptions, the sedimentation constant of 0.95×10^{-13} that he finds corresponds to a

minimum mol. wt. of 3300. The equiv. wt. on the other hand, whether calculated from the titration data given in the last paper or from the N content, is 170–190. In this paper some preliminary results on the hydrolysis of this high-molecular amine will be described.

Estimations of total sugar carried out by heating with orcin and H_2SO_4 [Pirie, 1936, 2] showed that AP contained 14% carbohydrate estimated as glucose. The constituent responsible for this reaction is still present in the amine and, as would be expected from the removal of considerable amounts of non-carbohydrate material, the apparent carbohydrate content is now increased (20–22%). The colour is developed rather more slowly in mixtures containing the amine than in control experiments containing the corresponding amount of glucose. Even after prolonged acid hydrolysis, e.g. 7 hr. at 100° with 5 *N* H_2SO_4 , the “carbohydrate” content of what little amine can still be isolated by precipitation with phosphotungstic acid is 20%. There is therefore good reason to look on this “carbohydrate” as an integral part of the amine molecule.

Reducing sugar estimations

Reducing sugar estimations have been carried out on hydrolysates by Somogyi's [1937] method, using glucose as the standard. The results of a typical experiment are given in Table I. If H_2SO_4 is used for the hydrolysis the destruction after the maximum reducing power has been attained is more marked, and fluids which have been decolored with charcoal after more than 2 hr. hydrolysis under these conditions give little or no reduction of the sugar reagent.

Table I

Time of hydrolysis at 100° with 3.6 <i>N</i> HCl min.	Reducing sugar estimated as glucose %	Tintometer Red Units per mg. of amine
0	2.2	3
10	6.5	8
20	9.2	9.5
35	11.1	13.5
64	13.5	16
90	15.6	19
140	19.3	21.5
210	17.8	16
440	16.5	12

Glucosamine and 3-amino-glucose may be estimated by this method and it is reasonable to assume that other amino-sugars would be also. The low value of the reduction is therefore evidence that free amino-sugars in a quantity at all comparable with the N content of the amine either are not formed during the hydrolysis or else are not stable. Even after prolonged hydrolysis, when the reducing sugar value has begun to fall again, from a third to a half of the original amine can be recovered by phosphotungstic acid precipitation. This suggests that the destruction or modification of the hypothetical amino-sugar is more rapid than the hydrolysis of the amine. Evidence that the amine does contain an amino-sugar will be presented later when the products of deamination are described.

The extreme stability of glucosamine towards acid hydrolysis is well known, whereas the known amino-sugars that do not have the amino-group in position 2 are relatively unstable and either decompose, e.g. 6-amino-glucose [Fischer & Zach, 1911], or condense to a non-reducing anhydride, e.g. 3-amino-altrose

[Levene & Meyer, 1923; Bodycote *et al.* 1934]. Levene & Meyer found that this condensation was most marked when strong acid was used for the hydrolysis. Some hydrolyses have therefore been carried out with 0.2 *N* HCl; during the first 30 hr. at 100° the reducing sugar value, measured either by the Somogyi method or by the reduction of ferricyanide, rises and reaches 20 % but it does not rise further on further hydrolysis. Nitric acid has been found useful in the hydrolysis of pentosans [Heuser & Jayme, 1923]; when it is used for the hydrolysis of the amine discoloration is completely avoided but the yield of reducing sugar is no greater. After 4–5 hr. at 100° with 1.4 *N* HNO₃ the reducing sugar has risen to 21 % and on further hydrolysis it falls again. At this stage the amino-N content, reckoned on the dry weight, is less than 1 %. Nitric acid hydrolysis is therefore useless for a study of the hypothetical amino-sugar. Prolonged hydrolysis with 0.2 *N* HNO₃ was no more successful.

It is well known that such amino-sugars as have been tested do not give colours when heated with orcin and H₂SO₄ to an extent in any way comparable with the N-free sugars. It is of interest therefore to consider whether the 20 % of "sugar" that is found in the amine by the orcin method is due to a slight capacity of the nitrogenous component to react with the reagent or to the presence of 20 % of normal sugar. The question is still unsettled but 5–10 % yields of an as yet unidentified alcohol-soluble material containing 60 % of total and reducing sugar can be isolated from the supernatant fluids obtained after precipitating the unchanged amine from hydrolysates with phosphotungstic acid.

Amino-sugar estimations

Acetylated glucosamine or chondrosamine, after heating with dilute alkali, will give a red colour with Ehrlich's pyrrole reagent [Elson & Morgan, 1933; Palmer *et al.* 1937]. It is probable that this reaction is due to the formation of an oxazole ring during the alkali treatment [Morgan, 1938]. The behaviour of the hydrolysed amine towards this reagent is interesting for it gives the colour after treatment with alkali alone to a greater extent than after treatment with alkali and acetylacetone. Glucosamine, chondrosamine and also 3-amino-glucose (prepared by the method of Freudenberg *et al.* [1926]) give no colour if the acetylacetone treatment is omitted. AP, formylated amine and amine liberated by the minimum acid hydrolysis give no colour or, in the last case, very little whether they are acetylated or not. The amount of colour that is given after further hydrolysis varies both with the intensity of the hydrolysis and the period of heating with alkali. The former relationship is illustrated in Table I. Samples of a solution of formylated amine in 3.6 *N* HCl kept at 100° were withdrawn after the intervals stated and evaporated to dryness *in vacuo* over solid NaOH. The evaporation takes a few hours and the residue is nearly neutral. Solutions containing 5–15 mg. per ml. were then made up, the latter concentration being used for the less thoroughly hydrolysed material. 0.2 ml. samples were then heated with 0.5 ml. 0.5 *N* Na₂CO₃ for 30 min. in a boiling water bath. Alcohol and a solution of *p*-dimethylaminobenzaldehyde were then added as in the method of glucosamine estimation of Palmer *et al.* [1937]. The figures given are the intensity of colour in Tintometer units when the final volume is 4 ml. and the cell 1 cm. deep. Under the same conditions, but substituting a solution of acetylacetone for the Na₂CO₃, about a third of this amount of colour is given at the optimum but the colour does not fall off again after prolonged acid hydrolysis. The amount of colour given by glucosamine, etc. is not greatly affected by the time of heating with acetylacetone so long as the heating

is continued for more than 15 min. With the materials derived from *Brucella*, on the other hand, the colour goes on increasing with further heating with Na_2CO_3 . Prolonged heating cannot be used quantitatively however for the fluid becomes discolored before the addition of Ehrlich's reagent.

From these results it is reasonable to conclude that one product of the hydrolysis of the amine is an amino-compound in some ways analogous to glucosamine. The results are compatible with the hypothesis that it is an amino-sugar with the amino-group in a position other than 2; on this hypothesis it is necessary to assume either that the amino-sugar is unstable towards acids or that it is easily converted into a non-reducing anhydride. As has already been stated, the isolation of apparently unchanged amine from solutions that have been hydrolysed so long that the reducing sugar value has passed the maximum is in favour of this hypothesis. It is probable therefore that it is merely a coincidence that the highest reducing sugar values that have been obtained are equal to the total sugar as estimated by the orcin method.

Hydrolysis under different conditions

Various other forms of hydrolysis have been tried both on AP and on the amine but the results have been no more satisfactory than those that have been described. 80 % acetic acid at 100° has little effect on the amine but reduces the serum precipitation end-point of AP by 90 % in 20 min., and there is a parallel fall in precipitability by $(\text{NH}_4)_2\text{SO}_4$. Some of the serological activity of AP remains after heating at 100° for an hour with $N/2$ NaOH but more vigorous hydrolysis destroys activity completely. There is, under these conditions, no liberation of inorganic phosphate and there is no precipitation of the phospholipin that we have referred to as PL 2 although PL 2 can be liberated by subsequent acid hydrolysis. Several attempts have been made to utilize the stability of AP towards alkalis in the extraction of the antigen or for separating it from agar but the method has not been found useful. On heating with alkali the free amine is gradually destroyed with the production of ammonia but no other product has been recognized.

Some attempts have been made to get identifiable products by hydrolysing the amine with strong H_2SO_4 in the cold. In one typical experiment 0.6 ml. of 70 % v/v. H_2SO_4 was added to 84 mg. of amine in 1 ml. of water and the solution was evaporated *in vacuo* over P_2O_5 in the cold. This technique was adopted because the dry amine dissolves very slowly in strong H_2SO_4 . After 6 hr. it was diluted by adding ice and a solution of phosphotungstic acid was added; the precipitate, after decomposition with baryta, gave 65 mg. of apparently unchanged amine and a few milligrams of alcohol-soluble waxy material, similar to that already mentioned [Miles & Pirie, 1939, 4], was isolated from the supernatant. The process was repeated and after 18 hr. hydrolysis only 1/3 was still precipitable by phosphotungstic acid. After removal of the phosphotungstic acid from the solution with baryta a brown scaly material was left which could not be separated into components with appreciably different physical properties or chemical constitution by fractional precipitation with alcohol. It is not precipitated by the other agents such as picric, picrolonic or flavianic acids which precipitate the unhydrolysed amine. The total carbohydrate content (orcin) was 13 % and the reducing sugar content (Somogyi [1937], using glucose as standard) was 14 % without any further hydrolysis; the amino-N content was 4.5 %. There is therefore no evidence for the production in quantity of a reducing amino-sugar. By this technique hydrolysis proceeds without much discoloration but sufficiently far for the material to give the Elson & Morgan reaction after

treatment with Na_2CO_3 . Under the conditions used in Table I it gives more colour, e.g. 40 Red Units per mg., than any other product that has been made from the amine.

Deamination of the amine

The amine can be conveniently deaminated under conditions similar to those of the Van Slyke amino-N estimation; the principal product of the reaction becomes insoluble in cold water after drying. From 46 mg. of amine dissolved in 0.3 ml. of water and 0.2 ml. of acetic acid and treated for an hour with 250 mg. of barium nitrite in 1 ml. of water, 50 mg. of a soft colourless flaky material were isolated by drying the fluid over NaOH after quantitative removal of the Ba with H_2SO_4 . This material was washed three times with 2 ml. quantities of water on the centrifuge and both the solid and the washings were dried. The former weighed 40 mg. and the latter 10 mg.; each contained 50 % of carbohydrate by the orcin method but the soluble material gives the strong brown colour in the cold that is characteristic of ketoses and some partly oxidized sugar derivatives. A suspension of the insoluble deaminated material dissolves if kept on a water bath for 1–2 hr. giving an opalescent solution; on drying, the material reverts to the insoluble state. Deaminated material is more easily hydrolysed by acids than the amine and with nitric acid there is no discoloration. After 1 hr. with 0.2 *N* HNO_3 the reducing sugar value rises to 40 % and to 50 % in 2 hr.; after 4 hr. it is still 50 %. These results are the best evidence so far available that the amine contains material properly called an amino-sugar, for by deamination the amine, with a carbohydrate content of 20 % by the orcin method (which does not estimate those amino-sugars that have been tested), is converted into a slightly larger quantity of material containing 50 % of apparent carbohydrate.

Several preparations have been made but it has not proved possible to get a higher carbohydrate content than this. The results are substantially the same if the more alcohol-soluble parts of a preparation made by gentle hydrolysis, or if amine that has been vigorously hydrolysed, e.g. 2 hr. at 100° with *N* acid, are used. In these cases, however, little or none of the preparation is insoluble in water although some of it may take 5–10 min. to dissolve.

Deaminated material has the following analytical composition: C, 43–45; H, 6.0–6.3; N, 1.5–3.1 %. The preparations have never been N-free although they have been made from products in which all the N appeared to be in the amino-form both by titration and by Van Slyke estimation. Deamination reactions, however, seldom lead to a simple product and the same side reactions which lead to this residual N are probably responsible for the low total sugar content of the preparation. No products of the hydrolysis of the insoluble material have yet been identified but the particles give a strong Schiff test and no reaction for pentose.

Deamination of glucosamine

The deamination product from glucosamine, chitose, has been prepared for comparison with the material that has just been described. When prepared by exactly the same method, a water-soluble gum results but, as Levene & Ulpts [1925] have found, when a solution of this gum in dilute, e.g. *N* HCl is distilled *in vacuo* at 40°, a large part of the preparation becomes insoluble and resembles the deamination product from *Brucella* closely in its physical properties. Both the soluble and the insoluble parts of the preparation give colours with the orcin reagent; the former corresponds to a carbohydrate content of 40 % and the colour comes up quickly in the cold. The insoluble flakes, like those from *Brucella*, stain in a few seconds with Schiff's reagent. Chitose in the insoluble

state gives as much colour on heating at 100° with the orcin reagent as its own weight of glucose; at 55° chitose flakes give more colour than glucose and this colour is a clear red like that given by agar and the aldehydo-sugars [Pirie, 1936, 2]. This resemblance is of interest for there is evidence that chitose is a 2:5-anhydro-sugar [cf. Schorigin & Makarowa-Semljanskaja, 1935, for references] and that part of the galactose in agar is in the 3:6-anhydro-state [Percival & Forbes, 1938]. Because of these observations 3:6-anhydro- α -methyl galactoside¹ [Ohle & Thiel, 1933] was tested by the colour reactions described for agar and was found to give more colour under the conditions used than any other substance that has been tried. The argument put forward by one of us [Pirie, 1936, 1] that these colour reactions give support to the hypothesis that agar is partly composed of aldehydo-sugars must therefore be abandoned.

Acetylation and deacetylation of AP

Acetylation experiments have been carried out with materials at four different levels of hydrolysis: AP, formylated amine, amine and hydrolysed amine. The products of these acetylations are in many ways similar although they can be distinguished. Pennell & Huddleson [1937] have acetylated products which they call the endoantigens of *Br. melitensis* and *abortus* by heating with sodium acetate and acetic anhydride and they have raised the acetyl contents from 2.3 to 7.7 %. The chemical relationship between their endoantigen and the preparations that we have called [PLAPS] and AP is at present too obscure for it to be profitable to discuss the connexion between their observations and ours.

AP acetylates easily if it is precipitated from solution with alcohol, washed twice with pyridine and then suspended in a mixture of five parts of pyridine and two parts of acetic anhydride. The loose flocculent precipitate changes quickly into a tenacious gum and this dissolves in a few hours at room temperature. After incubation for 10 hr. at 38° the solution is distilled *in vacuo* to small volume and dried over H₂SO₄ and NaOH. The residue is soluble in acetic acid, pyridine and hot alcohol but not in chloroform or ether; it is partly soluble in water or cold alcohol but is easily precipitated by traces of salt. The fractions that can be prepared by adding water and salt to a strong pyridine solution do not differ in acetyl content but they are presumably aggregated to different extents. The total volatile acid [Elek & Harte, 1936] corresponds to 32–34 % of acetyl; this substance however still contains 7.5 % of formyl estimated by the method described in the preceding paper. The formyl therefore accounts for 11.1 % of the "acetyl" found, leaving 20.9–22.9 % of presumably real acetyl. The equiv. wt. of a substance containing 7.5 % of formyl is 387 and the acetyl content of a diacetyl compound of this equiv. wt. would be 22.2 %. There is evidence therefore that this gentle acetylation has introduced two acetyl groups for each formyl originally present. These are presumably *O*-acetyl groups for they can be removed by the Zemlen [1926] technique.

10 ml. of alcohol were added to a solution of 72 mg. of acetylated material dissolved in 0.4 ml. of pyridine; there was slight precipitation but the solution cleared on the addition of 1 ml. of 6 % NaOH in 90 % alcohol. After a few seconds it became turbid and a precipitate soon separated. This was centrifuged off after an hour and dialysed, 35 mg. being recovered. The regenerated antigen had a formyl content of 9.5 % and could be precipitated, like AP, by 37 % saturation with (NH₄)₂SO₄ at room temperature but, again like AP, a higher concentration

¹ The preparation was kindly supplied by Dr D. J. Bell.

was needed for precipitation at 0° . With antisera it precipitated specifically to an end-point of $1:1.4 \times 10^6$; this end-point compares favourably with the $1:2.5 \times 10^6$ that is characteristic of good preparations of AP [Miles & Pirie, 1939, 2].

Acetylation of the degradation products of AP

The other three substances have been acetylated by adding 15 parts of pyridine to a 20–30 % solution of the amine or formylated amine in water and then 10 vol. of acetic anhydride. Formylated amine remains in solution after the addition of the pyridine but the other two precipitate; they all precipitate on the addition of the acetic anhydride. Amine and formylated amine dissolve completely at room temperature after a few hours whereas amine which has been vigorously hydrolysed, e.g. 2 hr. at 100° with 2 *N* HSO_4 , may need 12 hr. at 38° before all is in solution. After 24 hr. at 38° they are dried as before. All three dissolve in alcohol, pyridine and acetic acid but only acetylated hydrolysed amine is appreciably soluble in chloroform; they are all insoluble in ether. It has already been pointed out that the antigen in its various stages of disintegration is more soluble in cold ammonium sulphate solution than in warm solutions of equal salt content. These acetylated products behave in a similar manner. They dissolve slowly in cold water to give clear solutions but on warming the solutions become turbid and will precipitate in time. The process is perfectly reversible on cooling and can readily be demonstrated with a 1–2 % solution.

Acetylated formylated amine contains the expected 10–11 % of formyl whereas the others contain none; the percentages of total volatile acid, reckoned as acetyl [Elek & Harte, 1936], are 36 %, 31 % and 31 % respectively with the three substances. The formylated amine has therefore still got two acetyltable groups besides the formylated amine group, but after hydrolysis one of these appears to be lost, for the diacetylated derivative of a substance which, like the amine, has equiv. wt. 180 would contain 32 % of acetyl. The apparent acetyl content is raised neither by further periods of acetylation with pyridine and acetic anhydride nor by hydrolysing for 3.5 hr. during the acetyl estimation instead of for the 2.5 hr. recommended by Elek & Harte.

N-acetylated amine and *N*-acetylated hydrolysed amine are more easily hydrolysed by acids than the free amine if hydrolysis is measured by the production of reducing sugar. A typical experiment carried out with a 0.5 % solution of *N*- and *O*-acetylated hydrolysed amine in 0.8 *N* HCl at 100° gave the following reducing sugar values (Somogyi) after 15, 30, 60, 133 and 196 min.: 6.4, 13, 19.5, 25.5 and 26 %. It is clear that, although reducing sugar is produced more quickly under these conditions than under those in Table I, the final values are so nearly the same that little would be gained by using acetylated material in the study of hydrolysis of the amine. These results are in agreement with the interesting observation of Moggridge & Neuburger [1938] that *N*-acetyl methyl glucosaminide is more easily hydrolysed, with the production of reducing sugar, than methyl glucosaminide itself. In both cases the effect is only marked during the first stages of hydrolysis because hydrolysis proceeds both at the glucoside linkage and at the *N*-acetyl group. Free glucosamine is very stable in the presence of acids and Moggridge & Neuburger obtained an 80 % yield of reducing sugar. With the acetyl derivatives of the much less stable amine from *Brucella*, on the other hand, the highest value has been 26 % and further hydrolysis leads to the destruction that has already been described. Unsuccessful attempts have been made to hydrolyse *O*-acetylated *N*-formylated amine with formic acid,

but with 90 % acid, although there is little or no liberation of amino-N, destruction is more rapid than the production of reducing sugar; the course of hydrolysis with dilute formic acid is similar to the course with hydrochloric acid.

Acetolysis

It was hoped that stable and recognisable breakdown products would result from the acetolysis of these acetylated substances, but the yields of the only crystalline derivative that has been isolated from several attempts at the acetolysis of acetylated amine and *O*-acetylated *N*-formylated amine have been too small for any attempt at its identification.

To 40 mg. of acetylated amine, dissolved in 0.2 ml. of acetic acid, 1 ml. of acetic anhydride was added. All remained in solution although these substances do not dissolve if acetic anhydride is added to the solid. The solution was cooled and 1 ml. of acetic anhydride, to which 0.3 ml. of H_2SO_4 had been added with cooling, was poured in, causing the separation of resinous masses which soon dissolve. The mixture was left at 38° for a week by which time it had become brown. Nothing seems to be gained by using stronger acid or by leaving the mixture for longer at 38° in the first instance. It was poured on to a mixture of ice and 4 ml. of 2*M* sodium acetate solution and, after a few hours, extracted 3–4 times with its own volume of chloroform. The aqueous part was dried and extracted with pyridine but little or nothing was found in the extract. On evaporation the chloroform extract weighed 18 mg. and this material was extracted repeatedly with cold ether. The extracts, on evaporation, were partly crystalline and this crystalline material, weighing 1–3 mg., could be separated from the remainder by dissolving it in a small quantity of ether. It crystallised well from dilute alcohol after it had been separated from uncrystallizable material in this way. A pool of products from several experiments of this type was analysed and found to contain 3.9 % N (Dumas); 32 % acetyl and 12 % carbohydrate (orcin).

The part of the acetolysis products that is extracted by chloroform but is not soluble in ether will, on further acetolysis, give more of the ether-soluble material if the same technique is adopted. A more detailed study of this substance will be undertaken when more of the antigen is available.

DISCUSSION

On account of the elaborate series of partial hydrolyses, each followed by a fractionation, by which this amine has been derived from the bacterial antigen, itself a thoroughly fractionated product, it is reasonable to assume that the amine is as likely to be pure as any other molecule of colloidal dimensions that has been studied. Philpot's surprising observation that the product of these actions is centrifugally homogeneous lends further valuable support to this assumption. No products of the hydrolysis of the amine have yet been identified and it would seem that the methods that we have used in this work are unsuitable for the study of a molecule of this type for the losses on hydrolysis have been too great for useful results to be got with the quantities of material at present available. There is, however, reason to think that the amine is built up from only two or three different units and that, since its mol. wt. is only of the order of 3300, it should be possible soon to determine its structure.

Three facts have been established; the presence of 7.5–8.3 % of N all, or nearly all, in the amino-form; the presence of two hydroxyl groups for each N atom in preparations at the penultimate stage of hydrolysis; the presence of

material that reacts as carbohydrate by the orcin method. A polysaccharide built up from amino-hexose units would, if we neglect the small effect of the molecule's ends, contain 8·7 % of N, two hydroxyl groups for each N atom and would give no colour with the orcin reagent if we judge by those which have been studied. If the amine gives the orcin colour owing to the presence of a hexose in the molecule in the proportion of 1 hexose to 4-amino-hexose units it should, if it is a simple linear molecule and we neglect ends as before, contain 6·9 % of N and have three hydroxyl groups for each N atom. This hypothesis can therefore be excluded. Of the many other possible hypotheses two merit consideration; that the orcin colour is due to a substance smaller than a hexose, e.g. a triose, or that the amino-sugar from which the amine is apparently built is itself responsible for the action. This last hypothesis is in many ways the most attractive.

SUMMARY

The main antigen of *Brucella melitensis*, the amine and the *N*-formylated amine derived from it, have all been *O*-acetylated. The properties and inter-relationships of these substances is discussed.

The products of acetolysis of *N*- and *O*-acetylated amine are described.

The course of hydrolysis, measured by the production of reducing sugar, of the amine, of its *N*-acetyl derivative, and of the product of its deamination by nitrous acid are described, and the properties of the deaminated material compared with those of deaminated glucosamine.

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ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

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(with technical assistance by E. DODWELL)

FIVE specimens from *Brucella* were sent by Mr Pirie. They were examined in 0.5–0.95 % solution in 0.2*M* NaCl plus 0.01*M* phosphate of pH 7, in a Svedberg oil-turbine ultracentrifuge, by the “diagonal schlieren” method. The refractive increment of the sedimenting material was determined by integrating the curves on the original prints with the aid of a planimeter. As the areas are rather small for this purpose the tracing point of the planimeter was fitted with a lens, and the wheel was placed on a “Perspex” surface which gives perfect smoothness without slip. A Stanley “Allbrit” planimeter was used, set at 4:1 cm.², and the area was traced five times for each reading. Used in this way tests showed that the planimeter was accurate to 0.01 cm.² The base-line was chosen by drawing a straight line between two points on either side of the sedimentation boundary. In all runs so far studied, whatever the substance, the area has decreased markedly after the edge of the boundary has entered the lower half of the cell. We have evidence that this is probably due to convection, so we now base our determinations of refractive increment on the means of the earlier photographs. Tests with a specimen of serum albumin made by Dr Ogston gave with this procedure an area 99 % of the theoretical. With smaller or less homogeneous molecules the accuracy is less, but in our experience the extra run required to give the true base-line is seldom justified.

Table I

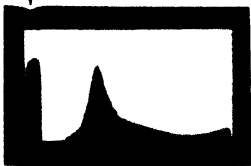



1 Photograph	2 Prep. no.	3 Material	4 $S_{20} 10^{-13}$	5 %	6 Homogeneity
Meniscus ↓ 	317A	AP, as usually prepared	ca. 20	ca. 65	Very poor, many faster particles
	317B	AP disaggregated by treatment with dodecyl sulphate	12.3	78	Good
Meniscus ↓ 	317E	317B reaggregated by precipitation with ammonium sulphate	ca. 50	ca. 57	Very poor, many faster particles
	346J	“Free amine”	0.95	50	Fair
	347A	“Formyl derivative of amine”	1.02	100	Good

Table I summarizes the results. Col. 4 gives the sedimentation constant in units of 10^{-13} . The figures in col. 5 are the refractive increment of the observably sedimenting material expressed as a percentage of the total refractive increment. The homogeneity given in col. 6 is only a rough guess and is decided by inspection of the photographs allowing qualitatively for the diffusion to be expected of a substance having the observed sedimentation constant. The photographs of 317A and 317E are complicated by various accidental factors and have been omitted.

The general conclusion from Table I is that the breakdown-products 317B, 346J, and 347A are more homogeneous than the original material 317A or the reaggregated material 317E. This is analogous to the fact that casein becomes homogeneous if the calcium is removed [cf. Philpot & Philpot, 1939]. The material not accounted for in col. 5 may be too low-molecular to sediment, or it may be high-molecular but so heterogeneous that its sedimentation curve is indistinguishable from the base-line.

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CCXIV. THE ACTIONS OF IODINE AND HYPOIODOUS ACID ON PEPSIN

By JOHN ST LEGER PHILPOT AND PERCY ARTHUR SMALL

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THIS paper gives experimental details of the work which we reported briefly at the Royal Society Discussion Meeting on "The Protein Molecule" [Philpot & Small, 1939]. We claim that inactivation of pepsin by iodine is sometimes due to action on groups other than tyrosine, and that these groups are not any of the ordinary amino-acids. Our evidence is that pepsin which has reacted with enough iodine to inactivate it still gives the full blue colour with Folin's reagent, whereas it should give less colour if the tyrosine groups had reacted appreciably.

EXPERIMENTAL

1. *General technique*

Pepsin was prepared as before [Philpot & Small, 1938, 2]. The concentrations quoted refer to this material, which contains about 10 % of water and 30–50 % of impurity [cf. Tiselius, 1938]. Peptic activity was measured by the original Anson-Mirsky method [Anson & Mirsky, 1932], except that the acid haemoglobin solution was made by mixing 1 vol. of methaemoglobin solution with 4 vols. of a solution containing 0.625 *M* H_2PO_4 and 0.167 *M* NaOH. This buffer gave a more reproducible *pH* than the HCl usually used. Methaemoglobin was used since oxyhaemoglobin gradually turns into it on storage. It was prepared as follows, partly following Adair & Adair [1934]. 450 ml. washed ox corpuscles were mixed with 132 ml. H_2O and 132 ml. purified ether, and shaken for 3 min. Then 57 g. NaCl were added, and the mixture shaken and centrifuged. The haemoglobin was treated with 25 ml. 10 % ferricyanide and 0.5 ml. octyl alcohol, shaken well and pressure-dialysed against distilled water. The concentration was adjusted so that on 250-fold dilution the blue colour given with Folin's reagent [Folin & Ciocalteu, 1927] was equal to that given by 0.055 mg. tyrosine per ml. The stock solution was stored under octyl alcohol in the cold room. These modifications caused little or no change in the activity unit, but seemed to give rather better reproducibility. The recent modifications by Anson [1938] appeared too late to be used in this work.

Folin's reagent [Folin & Ciocalteu, 1927] was used as follows. For peptic activities 3 ml. of the trichloroacetic acid filtrate were treated with 20 ml. 0.3 *M* NaOH and 1 ml. Folin's reagent. For Folin values of pepsin itself the trichloroacetic acid etc. were not present, though the pepsin was buffered, and a corresponding adjustment was made in the amount of NaOH. The colour intensity was compared in sodium light, after 30 min., with that of a glass filter or a mixture of cobalt nitrate and copper sulphate made to match the tint. As absolute values were not necessary calibration was usually dispensed with, so the units given are arbitrary and include dilution factors. In Fig. 3, however, the units are the same for both curves, so that the slopes can be directly compared.

2. *The action of iodine on pepsin*

Experiments were done under various conditions of pH, temperature, concentration and medium. Pepsin and iodine (in potassium iodide) were mixed and samples withdrawn from time to time. The unused iodine was titrated with bisulphite since the tetrathionate formed from thiosulphate reduces Folin's reagent. The titrated samples were then used for estimations of Folin value and peptic activity, after suitable dilution. In order to obtain reasonable velocities with moderate pepsin concentrations the iodine had to be in large excess. Hence the determination of iodine uptake was not very accurate, especially with the small volumes used and with bisulphite as titrant. The Folin values were more accurate, but unfortunately the greatest expected fall in Folin value was only 18 %. This arises from the facts that according to the analysis of Calvery *et al.* [1936] 11.5 % of the Folin value of pepsin should be due to tryptophan under our conditions, that only 10 of the 20 tyrosine groups are reactive (at least towards nitrous acid (cf. Philpot & Small [1938, 2]) and that under our conditions iodinated tyrosine has 50-60 % of the Folin value of tyrosine, mole for mole. For these reasons no attempt has been made at an exact kinetic analysis, though the time-courses of four experiments are recorded in Table I.

Table I. *Action of iodine on pepsin*

Curve (Fig. 1)	A			B		C		D	
pH	4.6			5.0		5.5		6.0	
Temp. ° C.	38			19		19		19	
Pepsin % (approx.)	0.25			0.12		0.12		0.12	
I ₂ normality	0.05			0.05		0.05		0.05	
<i>t</i>	I ₂	F	P	F	P	F	P	F	P
0	0.0470	41.1	50.5	52.5	46.9	51.0	47.2	49.5	51.4
2	0.0453	41.0	31.0	—	—	—	—	—	—
4	0.0446	40.0	28.2	—	—	—	—	—	—
6	0.0443	40.2	25.8	—	—	—	—	—	—
8	0.0436	39.8	22.0	—	—	—	—	—	—
10	0.0427	39.4	17.8	—	—	—	—	—	—
15	0.0423	39.6	14.3	—	—	—	—	—	—
20	0.0419	39.0	10.0	—	—	—	—	—	—
25	0.0419	39.0	8.0	—	—	—	—	—	—
30	0.0416	38.4	7.2	51.5	41.7	50.0	19.0	48.5	12.5
60	0.0395	37.5	6.9	50.5	37.2	49.0	11.5	48.0	6.0
90	0.0385	37.8	6.5	—	—	—	—	—	—
120	—	—	—	50.0	28.0	47.9	7.2	46.8	5.8
180	—	—	—	49.0	22.1	47.0	6.2	46.2	5.7
240	—	—	—	48.0	19.0	46.6	5.7	45.8	5.8

t Time from start in minutes.
I₂ Normality of iodine.
F Folin value, arbitrary units.
P Peptic activity, arbitrary units.

For our present purpose the most relevant information is obtained by plotting peptic activity against Folin value, each expressed as percentage of the original. This is done in Fig. 1. If the inactivation were due to iodination of one or more of the ten reactive tyrosine groups, all ten reacting at the same rate, one would obtain the line "10" in Fig. 1. If the inactivation were due to iodination of a single tyrosine group, which for some reason reacted much more rapidly than the others, one would obtain the line "1" in Fig. 1. Similarly two such groups would give the line "2". The experimental curves A-D represent the various conditions of temperature and pH recorded in Table I. Curve B, obtained at

19° and pH 5, is near the line "10"; but curves C and D, at pH 5.5 and 6, lie between the lines "1" and "2" until the inactivation is nearly complete. Curve A shows that at 38° the simple "10" hypothesis is untenable even at

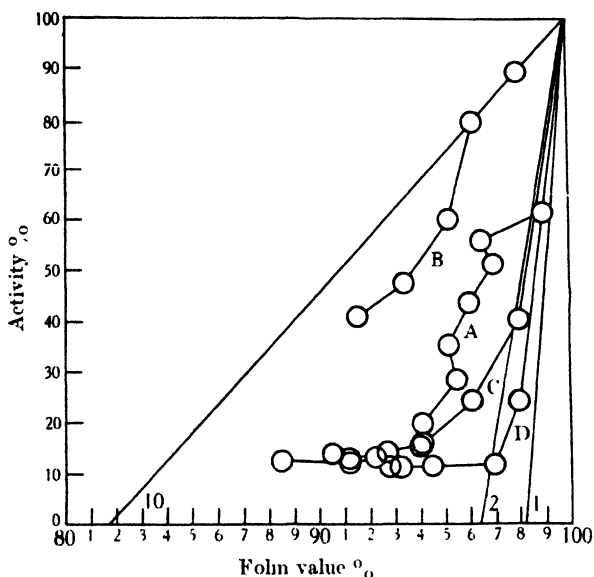


Fig. 1. Action of iodine on pepsin. For details see Table I and text.

pH 4.6. The general conclusion from these experiments is that at sufficiently high pH and temperature pepsin can be largely inactivated by iodine without iodinating more than two tyrosine groups.

3. The action of iodine on diazopepsin

The name "diazopepsin" refers to the yellow 50 %-active product of the action of nitrous acid, in which 50 % of the tyrosine groups have a diazo-group introduced *ortho* to the hydroxyl [Philpot & Small, 1938, 1, 2]. Since any further action of nitrous acid on the compound is extremely slow, and since the diazo-group can be altered in several different ways, even coupled with naphthylamine, without altering the activity (unpublished observations by P. A. Small) it was of interest to study the action of iodine.

The diazopepsin was prepared by mixing 20 ml. 2.4 % pepsin with 10 ml. 10 *M* acetic acid and 10 ml. *M* NaNO₂, leaving 3 days at 0° and then dialysing free of nitrite. It was stored at pH 4.6 in a darkened vessel in the cold room. It was handled with minimal exposure to light, to which it is very sensitive.

Table II gives the results of two experiments with iodine, and Fig. 2 gives the activity plotted against Folin value. The lines "1" and "10" are as in Fig. 1. The fall in activity is as rapid as with pepsin itself, but curve B, shows that at 38° much less than one tyrosine group per mol. is iodinated even when the activity has fallen to 20 %. The fall in activity must therefore be due to action on some other groups. These could be diazotyrosine groups, whose initial Folin value is practically zero: but no such action is known, and the indifference of the activity towards other changes in the diazotyrosine groups suggests that they play no part in it.

Table II. *Action of iodine on "diazopepsin"*

Curve (Fig. 2)	A		B	
pH	4.6		4.6	
Temp. ° C.	18		38	
Diazopepsin % (approx.)	0.6		0.6	
I ₂	0.05		0.05	
<i>t</i>	F	P	F	P
0	40.0	42.7	33.3	40.0
4	—	—	33.5	18.1
8	—	—	33.3	12.5
15	—	—	33.3	9.0
24	—	—	33.2	7.0
30	40.0	35.5	—	—
60	40.0	28.6	—	—
120	39.5	21.2	—	—
180	38.9	16.6	—	—
240	39.2	14.0	—	—
360	38.4	11.2	—	—
480	38.1	8.3	—	—

Symbols as in Table I.

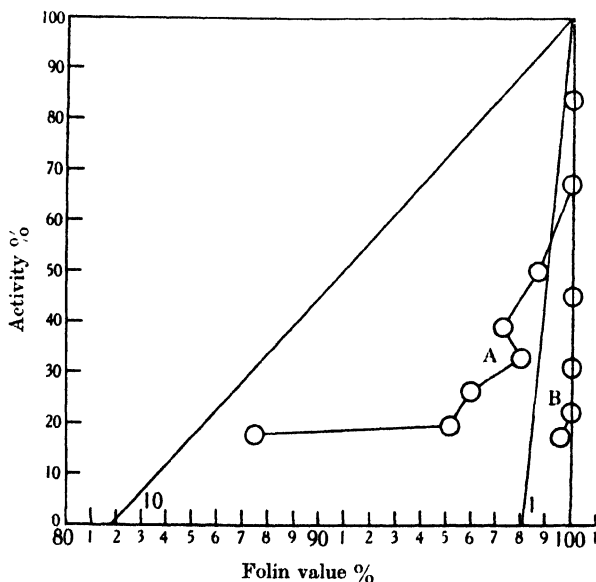


Fig. 2. Action of iodine on "diazopepsin". A at 18°; B at 38°.

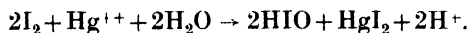
4. *The action of hypiodous acid on pepsin*

It was shown by Cofman [1920] that hypiodous acid acts extremely rapidly on phenols. Since iodine solutions are in equilibrium with hypiodous acid it is probable that the latter is an intermediate when the former acts on tyrosine. It was of interest to see whether the hypothetical non-tyrosine groups were acted on even more rapidly than tyrosine by hypiodous acid, as they should be if it was an intermediate for them as well.

The very high reaction rates involved here necessitated a change in technique which was advantageous for our purpose. Instead of adding a fairly large excess of iodine and titrating at various times, various small amounts of hypiodous

acid were added and allowed to react to completion. The activity and Folin values were then measured as functions of added hypoiodous acid, thereby eliminating time as a variable and avoiding the iodine titration. Under these conditions the residual concentration of the fastest reacting group present will be a linear function of the amount of added hypoiodous acid until it is low enough for more slowly reacting groups to compete.

Since HIO is fairly rapidly converted into IO_3^- and I^- it had to be generated *in situ*. The most convenient way of doing this was to add the appropriate amount of iodine and then an excess of mercuric acetate. The reaction was



After the action on pepsin was complete addition of excess iodide brought the $[\text{Hg}^{++}]$ to a very low value and prevented it from interfering with later operations. The iodide also regenerated iodine from any unchanged hypoiodous acid, and also from any iodate at *pH* below about 4, so that by testing for iodine at low *pH* it could be shown that the hypoiodous acid, if not in excess, had reacted completely in the time allowed. Controls showed that the reagents used without iodine did not affect the peptic activity but lowered the Folin value by about 4%. The 0.003 *N* I_2 used was made up of 0.0005 *M* KIO_3 , 0.004 *M* KI and 0.005 *N* H_2SO_4 . *x* ml. of it were added to 5-*x* ml. buffered pepsin or tyrosine and HIO was generated by adding 0.5 ml. of 0.03 *M* mercuric acetate. After 5 min. at room temperature 1 ml. of 0.2 *M* NaI was added, and the Folin value and activity were then determined as already described.

Fig. 3 shows the Folin values of pepsin and tyrosine plotted against amount of HIO. The point of interest is the initial slope, which is zero for pepsin but not

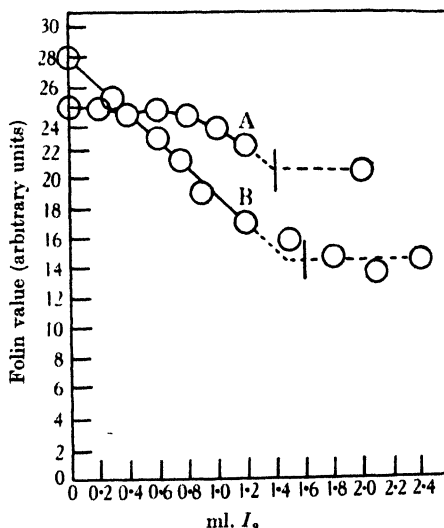


Fig. 3. The action of hypoiodous acid on pepsin and tyrosine at *pH* 5.4. A, pepsin 2.5 mg. B, tyrosine 0.18 mg. Beyond the vertical lines hypoiodous acid was in excess.

for tyrosine. This proves that the first action of HIO on pepsin is something other than iodination of tyrosine groups. The activity curve which is plotted with the Folin curve in our preliminary communication [Philpot & Small, 1939], shows that the same amount of HIO which leaves the Folin value unaffected causes nearly total loss of peptic activity.

5. *Studies on groups other than tyrosine*

In our preliminary communication a list was given of other groups which could not be concerned in the inactivation process in question. The evidence is as follows. Histidine, gelatin, asparagine and acetone reacted too slowly or not at all under our conditions. This rules out histidine, proline, hydroxyproline, amide, amino, carboxyl, peptide and simple ketone groups. Tryptophan and simple phenol groups are ruled out because if not masked they would react with nitrous acid, whereas the end-product from nitrous acid still contains the iodine-sensitive groups. Sulphydryl groups react much too rapidly with iodine, and cannot be detected in pepsin. The possible participation of disulphide groups has been difficult to eliminate. Cystine reacts fairly rapidly with iodine and very rapidly with hypiodous acid, the uptake in the latter case corresponding exactly to formation of cysteic acid. However, peptic activity is unaffected by hydrosulphite, which should reduce disulphide groups. Also, we have made various attempts to estimate the disulphide groups in pepsin before and after treatment with hypiodous acid. This involved special difficulties which have not yet been completely overcome; but the provisional conclusion is that not enough of the disulphide groups in pepsin can react at pH 5.4 to account for the whole of the HIO uptake involved in the inactivation process.

DISCUSSION

We do not claim that the above evidence for the existence of essential groups in pepsin other than ordinary amino-acids is complete, but we think it provides sufficient stimulus for an attempt to isolate the groups in question. It is perhaps worth noting that the optimal conditions for formation of thyroxine by an anomalous iodination of tyrosine [Ludwig & Mützenbecher, 1939] in proteins are very similar to our conditions for obtaining no initial fall in Folin value. We do not think that this can invalidate our conclusions, but the point might be investigated. Our results are nowhere in direct conflict with those of Herriott [1937], who claimed that inactivation of pepsin was due to iodination of tyrosine groups, since he did not use Folin's reagent to study the course of the reaction. Iodination of the tyrosine groups alone should certainly diminish the activity and might abolish it altogether; but we have no evidence on this point.

SUMMARY

1. When iodine acts on pepsin the initial ratio of fall in Folin blue value to fall in peptic activity decreases with increasing pH and temperature, reaching a very low figure at pH 6 and 38°.
2. The same occurs with "diazopepsin", in which the reactive tyrosine groups have previously been attacked by nitrous acid.
3. When pepsin is treated with small amounts of hypiodous acid the initial ratio of fall in Folin blue value to amount of hypiodous acid is much lower than for tyrosine, being practically nil at pH 5.4.
4. The hypiodous acid required to produce a just perceptible fall in Folin blue value is sufficient to destroy most of the peptic activity.
5. It is concluded that some groups other than tyrosine are concerned and reasons are given for excluding other amino-acids.

We thank the Department of Scientific and Industrial Research, the Nuffield Trust, the Medical Research Council and the Government Grant Committee

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CCXV. THE DISTRIBUTION OF THE FATTY ACIDS IN HALIBUT INTESTINAL OIL, WITH A NOTE ON THE PRESENCE OF FREE FATTY ACIDS IN THE INTESTINES OF FISH

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(Received 29 August 1939)

In a previous communication [Lovern & Morton, 1939] the suggestion was advanced that in halibut (and other species of fish which have large deposits of vitamin A in their intestines) vitamin A assists in the process of fat absorption and transference through the intestinal wall. If this view is correct, one would expect to find vitamin A esterified with all the fatty acids concerned; the amounts of the various esters being in the same proportion as the individual fatty acids of the fat being handled.

With the object of obtaining information as to the mode of esterification of vitamin A in this material, halibut intestinal oil was prepared in quantity. The oil is a complex mixture [Lovern *et al.* 1939] containing fat, free fatty acids, phosphatides, vitamin A and its esters, cholesterol and its esters and other substances. These components were separated as well as possible and the fatty acid composition of each fraction determined.

EXPERIMENTAL

Halibut intestines were collected by three commercial line-fishing vessels during the summer of 1938, and were brought back to Aberdeen in tins, packed among ice and salt. The average duration of a trip was about 10 days. The intestines were stored at -30° at Aberdeen until October, when they were extracted. The extraction process, including thawing, mincing, dehydrating with sodium sulphate and extracting with ether, took about 1 week. These data may be of importance in view of the high free acid content of the oil although further information is desirable as to the free fatty acid content of the functioning intestine (*vide infra*).

33,233 g. of intestines gave 1689 g. of "oil" (5.1 %). The oil was dark brown in colour and showed 4200 blue units. This vitamin content is somewhat lower than had been hoped for, and is attributed to the great preponderance of intestines from relatively small fish. The crude extract was treated with acetone, giving 1353 g. of soluble "fat" (80 %) and 336 g. of insoluble "phosphatides" (20 %). The acetone-soluble portion now showed 6500 blue units. Evidently some inhibiting substance had been removed along with the phosphatide fraction. The acetone-soluble "fat" was sent to Liverpool where it was found to contain about 73 % F.F.A. (mol. wt. taken as 340), about 6 % vitamin A and 21.5 % non-sap. (containing 29–31 % vitamin A).

As the oil was to be subjected to molecular distillation, for which process such a high free acid content is undesirable, it was taken up in ether and washed with the calculated amount of aqueous KOH. The result of this treatment was to yield about 65 % of free acids and 35 % of "neutralized" oil. The latter, however, still contained a considerable amount of F.F.A. This difficulty in removing free acid except with a large excess of alkali has been encountered before [Lovern *et al.* 1939] and is presumably due to the instability of soap in aqueous solution at pH less than about 8. Excess of alkali was, however, avoided as it was undesirable to risk any saponification of vitamin esters.

The "neutralized" oil was of a solid consistency: when it was dissolved in hot acetone much sterol (cholesterol) crystallized out as the solution cooled, and was removed by filtration.

The purified material was now liquid. To free it further from traces of phosphatides and gummy matter of the type previously shown to be present in such oils [Lovern *et al.* 1939] it was dissolved in 2 l. of acetone and cooled to -30° . 36 g. of gummy matter separated out. A repetition of this process, but using 2 l. of light petroleum instead of acetone, caused the removal of a further 6 g. of insoluble matter (not gummy, but solid at room temperature). Of the resulting oil (290 g.) half was analysed directly and half used for molecular distillation, which was carried out for us at the research laboratories of The British Drug Houses, Ltd. Table I gives particulars of the various fractions obtained.

Table I. *Details of molecular distillation*

Fractions	Wt. g.	Temp. ° C.	Vitamin A %	Non-sap. %
1	1.3	80	32.8	48
2	2.6	90	32.8	
3	8.3	100	36.8	
4	11.8	110	34.7	
5	11.0	120	30.7	
6	11.7	130	22.4	70
7	8.9	140	13.4	
8	5.5	150	8.7	
9	8.4	160	8.0	
10	6.1	170	10.7	
11	4.3	180	17.8	54
12	3.1	190	24.7	
13	7.3	200	27.3	
14	7.6	210	27.1	
15	7.4	220	23.4	
16	8.6	230	17.1	
Residue	ca. 30	—	8.3	49

Certain fractions were then combined for analysis of the constituent fatty acids, by the methods of ester distillation described previously [Guha *et al.* 1930; Lovern, 1934]. The groups so analysed were: fractions 1-5, fractions 7-10, fractions 11-16 and the residue. Fraction 6 was lost. The quantities of acids available were small and so the compositions arrived at must only be regarded as approximate. In addition to these samples, analyses were made of the free acids recovered in the alkali purification process, of the purified oil used for distillation and of the crude phosphatide fraction. The results of these analyses are given in Table II. The free acids contained 5 % of unsaponifiable matter (presumably entrained by emulsification) and the purified oil 48 %. The phosphatides, in addition to unsaponifiable matter, contained a considerable quantity of water-soluble impurities.

Table II. *Fatty acid analyses of various fractions*

Material	Saturated				Unsaturated				
	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C _{>22}
Phosphatides	3.3	26.5	9.3	0.2	5.0 (-2.3 H)	18.7 (-2.6 H)	15.6 (-4.0 H)	9.9 (-6.0 H)	11.5 (- ? H)
Free acids	1.1	13.6	1.5	—	6.4 (-2.5 H)	27.2 (-2.7 H)	22.6 (-6.4 H)	20.0 (-8.8 H)	7.6 (- ? H)
Neutralized oil	1.2	10.0	3.6	—	5.3 (-2.0 H)	28.2 (-2.8 H)	26.2 (-5.4 H)	16.0 (-7.3 H)	9.5 (- ? H)
Fractions 1-5	—	13	—	—	3	45 (-2.9 H)	27 (-6.0 H)	12 (-8.0 H)	—
„ 7-10	←—	3	→—		?	20 (-3.0 H)	46 (-5.3 H)	31 (-7.7 H)	?
„ 11-16	2	14	2	—	5	37 (-3.2 H)	23 (-6.0 H)	12 (-7.2 H)	5 (- ? H)
Residue	←—	8	→—		2	33 (-2.9 H)	29 (-5.2 H)	14 (-7.8 H)	14 (- ? H)

DISCUSSION

It can be seen from Table I that all the fractions contained appreciable quantities of vitamin A, but that its distribution was irregular, there being two groups of fractions with maximal contents of vitamin. The first group (say fractions 1-5) would contain predominantly free vitamin A and the second (say fractions 11-16) predominantly esterified vitamin A. At the same time there had evidently been considerable overlap in the fractions 7-10.

It can further be seen from Table I that all the fractions contained much unsaponifiable matter other than vitamin A. This would consist partly of cholesterol and partly of unidentified substances (including probably alcoholic substances which were free in the lower fractions and esterified in the later ones). The cholesterol in the lower fractions would also be free, whereas in the later ones it would be esterified. In fractions 7-10, the high content of non-vitamin unsaponifiable matter was probably largely free cholesterol.

Consideration of Table II reveals several points of significance. The neutralized oil had substantially the same fatty acid composition as the fatty acids washed out from it. Hence autolysis had been practically non-selective. On the other hand, the phosphatide fatty acids were appreciably different in that they contained more palmitic and stearic acids and less C₁₈, C₂₀ and C₂₂ unsaturated acids. This finding seems to suggest that these phosphatides do not play any direct part in the transport of absorbed fat in the intestine.

Turning to the distillation fractions, groups 1-5 and 7-10 both contained acids in different proportions from the original oil. However, in both these groups the acids must have been present largely as free acids and the differences would be expected owing to the molecular distillation process causing a greater preponderance of lower acids in the first fractions and of higher acids in the later fractions. If groups 1-5 and 7-10 were combined, the resultant fat would not be so different from the original fat. The acids of fractions 11-16 (the main vitamin ester fractions) were substantially the same as those of the original oil, as were those of the residue (also esterified matter, but with presumably a bigger proportion of sterol esters). The differences in fatty acid composition between fractions 11-16 and the residue are such as would be expected from the separating action of distillation.

It seems probable that the oil, as prepared in these experiments, contained little, if any, glyceride. Any triglyceride should have accumulated in the residue, but the amount of combined unaponifiable matter was sufficient to account for all the fatty acid present. This also applies to the other fractions. There remains the large proportion of free acid removed by alkali. This was certainly not all present originally as esters of vitamin, sterol etc., and the probability is that some of it was present in the functioning gut as glycerides, which were completely hydrolysed during the rapid post-mortem autolytic processes.

There is also the possibility, however, of appreciable quantities of free fatty acid being present in the living intestine. Hilditch & Shorland [1937] discuss the occurrence of free fatty acid in animal liver. The freshest halibut intestinal tissues so far extracted by us gave oils with free fatty acid contents ranging from 5.3 to 21.0 % [Lovern & Morton, 1939] and experiments on absolutely fresh halibut intestine have so far been held up by lack of material. Meanwhile, tests made on other species of fish (cod, conger eel, lythe, ling and dogfish) have so far not yielded oils with less than 5.0 % F.F.A. [see note]. Oils extracted only a few minutes after the death of the fish frequently contained up to 30 % F.F.A.

Whether or not the main part of the fatty acids in the functioning gut (and present as free acids in this sample) was present as glycerides or as free acids, the fact remains that vitamin A (and also cholesterol) was esterified with fatty acids in approximately the same proportions as the acids were present in the total extract. Whilst not a proof that vitamin A assists in the processes of fat absorption in halibut, this evidence supports such a hypothesis. If vitamin A (or cholesterol) does participate in fat transference, a certain amount of free fatty acid must be present at certain stages of the process, although, depending on the rapidity of some of the reactions, the concentration may never need to rise to a high level. With present evidence, there seems no reason to doubt the presence (or potential presence) of adequate concentrations of free fatty acid in the living gut.

A NOTE ON THE OCCURRENCE OF FREE FATTY ACID IN THE INTESTINES OF FISH [J. A. L.]

As all samples of intestinal oils so far examined have contained appreciable (and often large) proportions of free fatty acid, experiments have been made to ascertain whether such acid is normally present in the functioning intestine, or is entirely a post-mortem product. It would have been highly desirable to include halibut amongst the species studied, and a visit to the Shetland Isles on the research vessel attached to the Torry Research Station was made for this purpose. Unfortunately, the presence of very large numbers of dogfish on the fishing grounds prevented the catching of any other species in these waters. The other fish studied were caught near Aberdeen.

In these experiments various procedures have been followed. In the first series, on cod, the fish were brought back ungutted by local yawls and were only just dead when landed. The fish were gutted as rapidly as possible, the stomachs removed from the intestines and the food residue washed out, either by forcing water through the gut, or by slitting it open and washing in water. The pyloric caeca were sometimes worked up separately but gave values identical with those of the intestines. Following the washing, the intestines were minced, desiccated with anhydrous sodium sulphate and extracted with ether. The whole batch of intestines was collected and treated together, involving a certain time lag for some of them.

In the next experiment, intestines were removed from live cod on the research vessel, washed, and at once placed in a large volume of ethyl alcohol. On return to Aberdeen they (and the alcohol) were worked up for fat and the fat tested for free acid.

Following on this, experiments were carried out wholly at sea. In the first of these, on dogfish, the intestines were removed from living fish, a number collected, slit open and washed, then minced, desiccated with sodium sulphate and extracted with ether. As it was becoming evident that great speed was of the utmost importance, the method was now altered. The intestine from one fish at a time was dealt with, instead of accumulating a number before washing and mincing. After mincing the intestine was at once mixed with a large volume of acetone, which was thought to be a more rapid method of dehydrating. The effect of this more rapid technique can be seen from Table III but it still appears that there must be some free acid in the living gut, although there may, of course, be differences in amount from species to species.

Table III. *Free fatty acid in the intestines of fish*

Species	Time between death and beginning of desiccation	Treatment	F.F.A. in oil %
Cod	2-3 hr.	Bulk washing and sodium sulphate	30.0
"	2 hr. 15 min.	" "	30.1
"	1 hr. 20 min.	" "	27.8
"	5-10 min.	Single washing and alcohol before mincing	23.2
Dogfish	5 min.	Bulk washing and sodium sulphate	23.6
"	10 min.	" "	18.3
"	1-2 min.	Single washing and acetone after mincing	7.4
Conger eel	1 min.	" "	5.0
"	1 min.	" "	6.1
Lythe	1 min.	" "	7.7
Ling	1 min.	" "	9.6

SUMMARY

A large quantity of halibut intestines was extracted with ether and the resulting oil was partially separated into its components, the processes used being alkali-washing, acetone precipitation and crystallization and molecular distillation. The products separated for examination included free fatty acids, phosphatides, vitamin and cholesterol esters and a "neutralized" oil.

The fatty acids recovered from these products were quantitatively analysed and it is shown that both vitamin A and cholesterol were esterified with all the various fatty acids present, the amounts of the different esters being proportional to the relative amounts of the acids present in the main fatty extract. This evidence is in harmony with the hypothesis that vitamin A assists in fat absorption processes.

The fatty acids of the phosphatide fraction were appreciably different from those of the main fat fraction and it seems unlikely that this phosphatide was directly concerned in fat absorption.

The probable presence of free fatty acid in the living intestine is indicated and the bearing of this on vitamin-assisted fat absorption is discussed.

Our thanks are due to the Medical Research Council for a grant in aid of the expenses of the work (R. A. M.) and to Dr F. H. Carr and Dr T. H. Mead, of The British Drug Houses, Ltd., for kindly carrying out the molecular distillation.

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CCXVI. THE PREPARATION OF CANAVANINE FROM *CANAVALIA OBTUSIFOLIA*

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(Received 1 September 1939)

OUR interest in canavanine arose under the same circumstances which in the first instance led to its discovery by Kitagawa & Tomita in jack bean [1929]. In studying some new sources of urease it was found that the seeds of *Canavalia obtusifolia* resembled jack bean not only in possessing high urease activity, but also in giving rise to very large amounts of "extra" urea on incubation with whole blood or liver tissue [Damodaran & Sivaramakrishnan, 1937]. The aqueous extract of the seed meal gave the ruby-red reaction with irradiated sodium nitroprusside characteristic of canavanine and after some preliminary trials the amino-acid was isolated in good yield from this new source.

Difficulty was experienced in obtaining homogeneous well crystallized preparations by adhering strictly to the procedure described by Kitagawa & Yamada [1932]. Their method consisted in extracting the fat-free jack bean meal with aqueous alcohol, concentrating the extract to a thick syrup and treating it with absolute alcohol. The crude canavanine thus precipitated was redissolved in water and converted into canavanine flavianate. From the twice-recrystallized flavianate the free base was liberated by barium hydroxide, taken up in aqueous alcohol and allowed to crystallize. It was found by these authors that the amino-acid thus obtained was contaminated with some persistent impurity. In a later publication Kitagawa [1937] therefore recommended the destruction of the impurity by digesting the first crude canavanine precipitate for several hours with 10 % HCl. Gulland & Morris [1935], who also seem to have experienced the same difficulty, suggest the purification of the base liberated from the flavianic acid salt by conversion into the rufianate.

We have found that the simplest procedure for obtaining satisfactory yields of the pure product is to introduce a preliminary treatment of the crude canavanine solution with basic lead acetate.

EXPERIMENTAL

The seeds were freed from husk, ground finely and defatted with light petroleum. 1 kg. of the fat-free meal was extracted three times with 50 % alcohol (1 l. alcohol per 250 g. meal with 2 hr. shaking each time). The extracts were combined, concentrated under reduced pressure to a syrup (400 ml.) and poured into about 5 l. of absolute alcohol with vigorous stirring. The crude canavanine was precipitated as a viscous mass. After standing in the refrigerator overnight the alcohol was decanted from the precipitate of crude canavanine, the sticky solid redissolved in 300 ml. of distilled water, and again treated with absolute alcohol as before. The reprecipitated material was dissolved in about 3½ l. of water and treated with a saturated solution of basic lead acetate until

precipitation was complete. The precipitate was centrifuged off and washed with small quantities of warm water.

The combined filtrates were made acidic to Congo red by the addition of dilute H_2SO_4 ($N/5$) and after filtration from the lead sulphate, treated with excess of flavianic acid solution (200 g. in 500 ml. of water) with vigorous stirring. A heavy orange-yellow precipitate consisting of microscopic needles appeared in a few minutes. The mixture was left in the refrigerator overnight, filtered at the pump and the precipitate washed with ice-cold water. It was recrystallized twice from $1\frac{1}{2}$ l. of hot water. M.P. 212° , after previous browning at 190° .

The flavianate was dissolved in about 3 l. of hot water and a saturated solution of $\text{Ba}(\text{OH})_2$ was slowly added with vigorous stirring until the mixture was strongly alkaline to litmus. The barium flavianate was filtered at the pump and washed three times with dilute $\text{Ba}(\text{OH})_2$ solution, the precipitate being ground up each time with $\text{Ba}(\text{OH})_2$ before being returned to the filter. Excess of Ba from the combined filtrates was removed by the addition of $N/10$ H_2SO_4 to pH 7. The Ba-free filtrate was golden yellow in colour and contained traces of flavianic acid. It was concentrated under reduced pressure to a volume of 600 ml., boiled with animal charcoal for a few minutes which removed the last traces of flavianic acid, the colourless solution freed quantitatively from H_2SO_4 using $N/10$ solutions of $\text{Ba}(\text{OH})_2$ and H_2SO_4 and the filtrate concentrated under reduced pressure in an atmosphere free from carbon dioxide, to a volume of 300 ml. Concentration to a syrup is inadvisable as it renders crystallization difficult. To the solution obtained as above three volumes of 95% alcohol were added and the mixture was left at 0° for a day. The canavanine which crystallized out in irregular prisms was filtered at the pump, washed with a small volume of ice-cold water and then with cold 75% alcohol. A second lot of crystals was obtained by concentrating the mother liquor. The yield obtained was usually more than 20 g.

To follow the course of the fractionation quantitatively, in one experiment the total N and the urea produced by the action of liver extract was determined on the solution obtained at each stage. The canavanine present was calculated on the assumption that it was the only substance present responsible for urea formation. The results are given in Table I. The actual yield of crystalline canavanine was 23.5 g.

Table I

	Total-N (g.)	Urea-N (g.)	Canavanine present (g.)
Alcoholic extract from 1 kg. (dry wt.) of seed meal	21.56	7.42	46.3
Filtrate after treatment with basic lead acetate	15.38	6.44	40.1
Filtrate after decomposition of flavianate	10.36	5.13	32.0

For analysis the product was recrystallized three times from aqueous alcohol and dried at 100° *in vacuo* over P_2O_5 . The M.P. of the recrystallized sample was 183° . (Found: C, 35.38; H, 6.82 (Weiler & Strauss); N, 30.61% (micro-Kjeldahl). Calculated for $\text{C}_5\text{H}_{12}\text{O}_3\text{N}_4$: C, 34.1; H, 6.82; N, 31.8%.) Gulland & Morris [1935] give C, 34.2; H, 6.8; N, 30.7%. In the two papers of Kitagawa available here no elementary analysis is given. We find amino-N (micro-Van Slyke), 50.6% of the total N in 5 min. at 30° .

SUMMARY

From the seeds of *Canavalia obtusifolia* the amino-acid canavanine can be readily prepared in pure condition in yields amounting to above 20 g. per kg. of the fat-free seed meal.

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CCXVII. ON THE SPREADING OF DIFFERENT HAEMOGLOBINS, MUSCLE HAEMOGLOBINS AND CYTOCHROME *c*

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(Received 10 June 1939)

STUDIES of Gorter & Grendel [1929] and Hughes & Rideal [1932] have shown that proteins, although mostly soluble in water, are usually almost completely absorbed in a homogeneous layer on the surface, provided that this is large enough. The thickness of this protein layer is, if the layer is under a small pressure (2 dynes per cm.), of the order of the length of one amino-acid, i.e. c. 7 Å. This is far less than the diameter of a protein molecule. Therefore the molecule must be unfolded so that every amino-acid can have its place on the water surface. At a pressure of more than 2 dynes the hydrocarbon chains of the amino-acids are nearly vertical to the surface. At a lower pressure they are flat on the water. The time necessary for complete unfolding to occur on dilute buffer solutions is short when the *pH* of the solution is the same as the isoelectric point of the protein. On diluted buffer solutions of other *pH* values the unfolding of the protein molecules proceeds far more slowly; some of the protein dissolves in the fluid of the trough and in most cases homogeneous monolayers are formed extremely slowly [Gorter & Philippi, 1934]. On more strongly acid and alkaline solutions (*pH* 1 and 13) monolayers are immediately formed.

The time taken by a protein molecule to unfold at its isoelectric point varies greatly, even in closely related proteins [Jonxis, 1935; Brinkman & Jonxis, 1935; 1937]. The results to be presented deal with "unfolding time" and area measurements of different haemoglobins, muscle haemoglobin and cytochrome *c*, and the influence of concentrated salt solutions, urea and reducing substances on the spreading of these proteins.

MATERIALS AND METHODS

A Langmuir trough with Gorter's modifications [Gorter & Seeder, 1935] was used to measure the surface areas. The *pH* of the trough solutions was determined with indicators. The protein solutions were applied on the surface by means of a micro-pipette as used by Gorter & Seeder [1935]. Whilst blowing out the protein solution the pipette is slowly moved over the surface of the liquid in the trough, so that the protein solution comes into better contact with the surface. Mitchell [1937] has found that with this method gliadin only forms its maximum surface when the concentration of the gliadin solution is less than 0.1 mg. per ml. I therefore tested the influence of the concentration of the protein solution on the maximum area and the time after which this area is reached. The protein I used was horse haemoglobin; it was placed on 0.1 *N* HCl and on a 0.003 *M* phosphate buffer solution (*pH* 6.8).

Between concentrations of 25 and 0.05 mg. per ml. there was no influence of the concentration of the protein on the maximum area or on the time in which this area was reached.

In order to have sufficient protein on the surface it was necessary to use greater quantities of the more diluted solutions (50 μ l. for a concentration of 0.5 mg. per ml., 500 μ l. for a concentration of 0.05 mg. per ml.).

The concentrated solutions (10–25 mg. per ml.) must be blown out of the pipette very slowly because otherwise a part of the protein does not remain on the surface, but sinks to the bottom of the trough. All area measurements were done at room temperature.

Preparation of the proteins

Horse haemoglobin. (a) Horse blood was washed three times with a 0.9 % NaCl solution. To 100 ml. red blood corpuscles there were added 30 ml. distilled water and 30 ml. washed ether. After shaking for 5 min. 13 g. NaCl were added. After centrifuging, the clear haemoglobin solution was dialysed against distilled water in the ice chest.

Alcohol was added at a low temperature till the concentration was 20 %. The solution was left in the ice chest and the next day the crystals could be removed and washed with 20 % alcohol. Then they were dissolved in water by adding a few drops of *N*/10 NaOH at 39° till the fluid became dark and clear. After addition of the same amount of *N*/10 HCl the haemoglobin solution was placed in the ice chest and the next day a mass of crystals could be centrifuged off. This was repeated three times. Finally the haemoglobin crystals were dissolved in a diluted alkaline phosphate buffer solution and dialysed against a diluted phosphate buffer solution of pH 6.8. The spreading properties of this haemoglobin solution were just the same as those obtained with a solution prepared in the following way.

(b) The horse blood was washed three times with 0.9 % NaCl. The red blood corpuscles were haemolysed with the same volume of distilled water. After adding 0.25 volume *M*/10 phosphate buffer solution the solution was centrifuged to remove the precipitate of stroma proteins. The clear haemoglobin solution was diluted with distilled water to a concentration of 5 mg. per ml. The latter method has the advantage of taking less time. This is very important because the spreading properties of a haemoglobin solution change when the haemoglobin solution is some days old, although it has been stored in the ice chest. Small amounts of stroma proteins and lipins seem to have no influence on the spreading properties of haemoglobin, so it is not necessary to remove them completely.

The concentration was determined first gasometrically by the Van Slyke method, later colorimetrically.

Native globin was made and recombined with haem to haemoglobin according to the method of Anson & Mirsky [1930].

Muscle haemoglobin was prepared by the method of Theorell [1934] and was twice recrystallized.

Cytochrome c was prepared by the method of Keilin & Hartree [1937].

RESULTS

The relation between area and pressure for a monolayer of horse haemoglobin on 0.003*M* phosphate buffer solution (pH 6.8) after the maximum area is reached is given in Fig. 1.

In its compressed state 1 mg. of horse haemoglobin covers (extrapolated to zero pressure) an area of 9400 cm.² This gives for one molecule an area of 10,560 Å.² A small part of this area (4 × 70 × sq. Å.) will be occupied by the four

haem groups of the molecule [Adam, 1938]. One molecule of horse haemoglobin is built up from 576 amino-acids [Bergmann & Niemann, 1937], therefore the area per amino-acid on the water surface will be 17.8 sq. Å. This value agrees very well with the results of Hughes & Rideal [1932] and Philippi [1934], who found for gliadin and ovalbumin under the same circumstances an area of 16.5 and 17 sq. Å. respectively per amino-acid. The side-chains under these conditions are up in the air or down in the water and only the polypeptide chains will be on the surface.

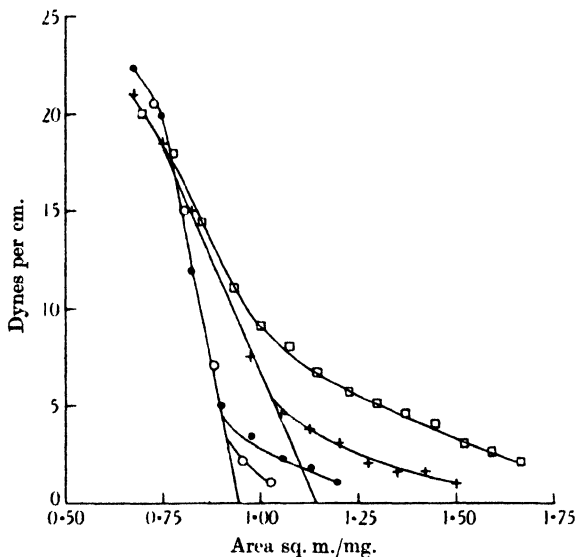


Fig. 1. Surface pressure curve obtained with monolayers of different haemoglobins.
 • Horse Hb at pH 6.8. ○ Cow Hb at pH 6.8. + Horse Hb at pH 1. □ Muscle Hb at pH 1.

I investigated haemoglobins of man, cow, dog, sheep, rabbit, cat and rat (both adult and foetal forms), and found that after the moment of reaching the maximum area the same area-pressure curve was obtained in the compressed state; when extrapolated to zero pressure the area was about 9400 sq. cm. per mg. The same curve was also found for the native globins prepared from these different haemoglobins. This shows that the total numbers of amino-acids of all these haemoglobins are the same per g., and if the mol. wt. is the same in the surface as in the interior of the solution, there will be the same number of amino-acids per per mol., i.e. 576.

At a lower pressure, below 3 dynes per cm., there are small differences between the surface pressure curves of the haemoglobins of different species (see Fig. 1). At these lower pressures the hydrocarbon chains of the amino-acids are mostly horizontal on the water surface and in this position the area is determined not only by the number, but also by the nature of the amino-acids.

On 0.1N HCl and on 0.1N NaOH monolayers are somewhat more compressible. The surface-pressure curves for pressures higher than 3 dynes per cm. are the same for all haemoglobins.

In Fig. 2 the relation between area and time at pH 6.8 is shown. The "unfolding time", after which the maximum area is reached, is, for human haemoglobin 5 min., for horse haemoglobin 18 min., for cow haemoglobin 35 min.

The time is independent of the way in which the haemoglobin has been prepared. Small amounts of impurities, such as stroma proteins and lipins, have no influence on this "unfolding time".

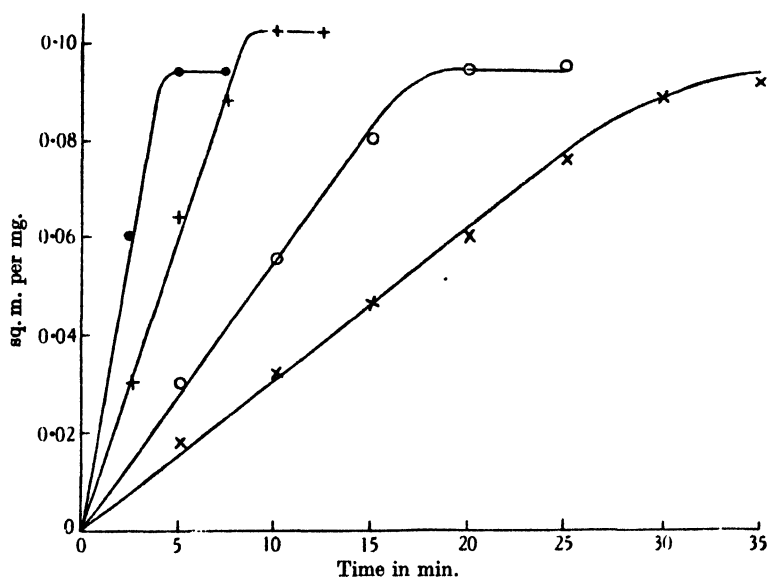


Fig. 2. Influence of the time on the area of monolayers of different haemoglobins.
 • Human Hb. ○ Horse Hb. × Cow Hb. + Horse Hb on 25 % urea at pH 6.8.

Higher salt concentrations of the liquid in the trough greatly increase the spreading tendency of proteins on the acid and alkaline sides of their isoelectric points [Gorter *et al.* 1932], but at the isoelectric point the concentration and the nature of the ions in the solution have no influence on the unfolding time; except on very concentrated solutions, where the unfolding time is somewhat smaller (see Table I).

Table I

Phosphate concentration	pH	Unfolding time in minutes	
		Horse Hb	Cow Hb
0.001 <i>M</i>	6.8	16	35
0.010 <i>M</i>	6.8	18	35
0.100 <i>M</i>	6.8	16	33
0.200 <i>M</i>	6.8	12	30
0.010 <i>M</i> + 0.001 <i>M</i> glutathione	6.8	17	35

Probably these differences in unfolding time are the results of different intramolecular linkages (especially S—S linkages) between the cysteine groups. No correlation could, however, be found between the amount of sulphur [Roche, 1936] and the unfolding times of haemoglobin molecules of different species. Other proteins, although containing S—S linkages, are immediately unfolded at their isoelectric points. This suggests that the S—S linkages do not seriously hinder the unfolding of a protein molecule. After keeping the haemoglobin solution a few days, the unfolding time decreases; it is therefore necessary to work only with fresh haemoglobin.

Fig. 3 shows the influence of the pH on the formation of monolayers of haemoglobin. Between pH 3–6 and 7.5–10 monolayers on 0.003*M* buffer solu-

tions are very imperfectly formed, most of the protein dissolving in the liquid of the trough. On concentrated buffer solutions (0.1*M*) the maximum spreading is almost immediately reached on solutions of pH 3–6 and 7.5–10, the unfolding

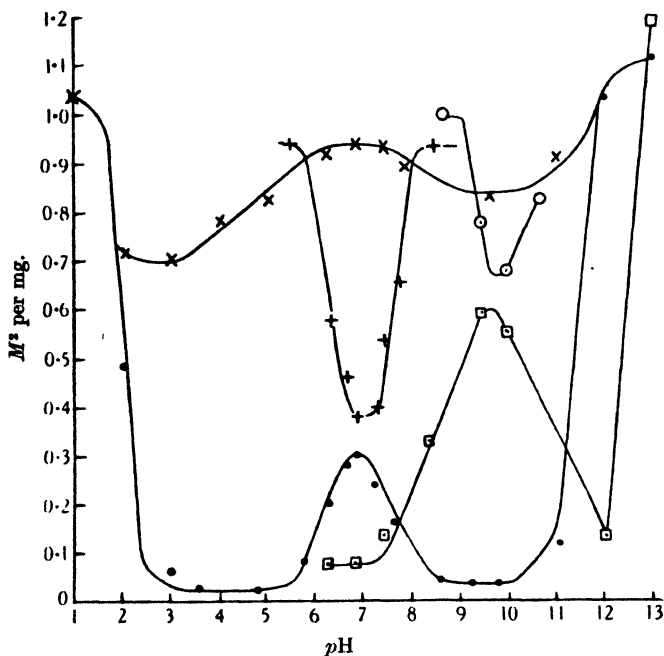


Fig. 3. The effect of pH on the spreading of horse haemoglobin and cytochrome. Time 5 min. • Horse Hb on diluted solutions. + Horse Hb on 0.2*M* phosphate buffer solutions. × Globin on diluted solutions. ○ Cytochrome on diluted solutions. □ Cytochrome on concentrated solutions.

time at the isoelectric point being the same as that on diluted solutions. Thus on concentrated solutions we find a minimum spreading tendency at the isoelectric point [Philippi, 1936; Danielli, 1938]. Native globin forms monomolecular films very easily. At pH 6.8 the maximum area is almost immediately reached (see Fig. 4). Haemoglobin synthesized from native globin and haem has the spreading properties of globin. By splitting off the haem the structure of the molecule must so be changed that the unfolding of the molecule proceeds more easily. In the synthesized haemoglobin the original structure is not restored. That synthesized haemoglobin is not identical with the normal haemoglobin is known from the work of Hill [1939], who found that the dissociation curve of synthesized haemoglobin is not of S-shape, but is a simple hyperbola.

The influence of urea on the spreading of haemoglobin

Burk [1932], Burk & Greenberg [1930] and Steinhardt [1938] have shown that in concentrated solutions of urea (25%) the molecular weight of horse haemoglobin is halved or even further diminished. The change in molecular weight was confirmed by Wu & Yang [1932] who showed that horse and ox haemoglobins are dissociated in concentrated urea solutions, but that the haemoglobins of sheep and dog are not. What, now, is the influence of urea on the spreading properties of haemoglobin?

Horse haemoglobin was dissolved in a 2 % urea solution and brought on to the surface of a 0.003*M* buffer solution, which contained 2 % urea. This concentration of urea had only a slight influence on the spreading. The surface pressure curve remained unchanged and the unfolding time was decreased from 18 to 15 min. The influence of more concentrated urea solutions, in which the haemoglobin dissociates, is greater. The surface pressure curve is flatter (Fig. 4). At greater areas, more than 1 sq. m. per mg., the pressure is higher and the layer more compressible. At pressures higher than 15 dynes/cm. the surface pressure curve is the same as on normal buffer solutions. This seems to indicate that on the concentrated urea solutions the side-chains are attracted by the surface. For horse haemoglobin the unfolding time is reduced from 18 to 9 min. For sheep haemoglobin, which is not dissociated by 25 % urea, the surface pressure curve is the same as that of horse haemoglobin and the unfolding time is diminished from 30 to 17 min. This indicates that the dissociation unto molecules of half the normal size is not the cause of the diminution in the infolding time.

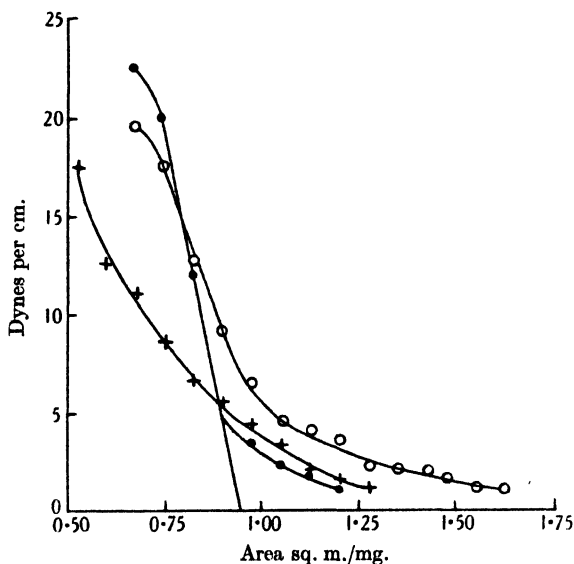


Fig. 4. Area pressure curve of horse haemoglobin and cytochrome and the influence of urea.
 • Horse Hb at pH 6.8. ○ Horse Hb on 25 % urea at pH 6.8. + Cytochrome at pH 9.7.

The spreading of reduced haemoglobin

When a small amount of sodium hydrosulphite is added to the fluid in the trough, the maximum spreading is reached after 3 min. It does not make any difference whether reduced or oxyhaemoglobin is used. In both cases the maximum spreading is reached after 3 min. It is therefore improbable that the shorter unfolding time of haemoglobin on a reduced liquid is to be accounted for by the heat developed when the oxyhaemoglobin is reduced by the hydrosulphite.

The surface pressure curve is the same as that of oxyhaemoglobin on non-reducing solutions. If more haemoglobin solution is brought on to the surface some minutes later, when the sodium hydrosulphite in the trough has been oxidized by the air, it again takes 15 min. to reach the maximum spreading.

This shows that the unfolding time for reduced haemoglobin is far shorter than that for oxyhaemoglobin.

I have tried to repeat this experiment with other reducing substances (Stokes' solution), but the reducing power was already exhausted before the haemoglobin could be brought on to the surface. Haurowitz [1938] has found that there are differences in the forms of the crystals of reduced and oxyhaemoglobin probably due to differences in positions of the haem groups in the molecule. The differences in unfolding time between reduced and oxyhaemoglobin also suggest that there are differences in structure between reduced and oxyhaemoglobin. The resistance of the oxyhaemoglobin molecule to unfolding must be the result of special properties of the oxygenated haem groups, because reduced haemoglobin and globin are almost immediately unfolded.

The spreading of methaemoglobin

When some drops of ferricyanide are added to the solution in the trough the Fe^{++} of haemoglobin is oxidized to Fe^{+++} and methaemoglobin is formed. This change has no influence on the spreading. The surface pressure curve and the unfolding time are the same as those of oxyhaemoglobin.

The spreading of muscle haemoglobin

Muscle haemoglobin differs from the blood haemoglobin of the same species in its lower molecular weight (17,000) [Svedberg, 1939], its hyperbolic dissociation curve and a small difference in absorption spectrum. It is far more soluble and is precipitated only by saturated ammonium sulphate. Its spreading properties are also different from those of the blood haemoglobin. Between pH 4 and 11 monolayers are hardly formed at all, even on concentrated buffer solutions; the solubility seems to be too great. On more acid and alkaline solutions monolayers are formed, but under these circumstances the muscle haemoglobin is denatured. No differences between the spreading properties of horse and cow muscle haemoglobins could be detected. The globin prepared from muscle haemoglobin has, on buffer solutions with a pH between 3 and 11, the same spreading properties as the globin of blood haemoglobin. This indicates that the total number of amino-acids per Svedberg unit is the same as that of blood haemoglobin; viz. for a molecular weight of 17,000, 144 amino-acids. On solutions of pH 1 and 13 the surface pressure curve is flatter than that of blood haemoglobin, so there must be some differences between the two globins.

The methaemoglobin formed from muscle haemoglobin has the spreading properties of muscle oxyhaemoglobin, but reduced muscle haemoglobin immediately forms monolayers.

When a small amount of sodium hydrosulphite is added to a phosphate buffer solution (pH 6.8) a monolayer of reduced muscle haemoglobin is formed and the normal maximum area (9400 cm.² per mg.) is reached in 5 min. When the same experiment is repeated after some time the hydrosulphite is oxidized and the muscle oxyhaemoglobin will not form monolayers. This demonstrates again that the condition of the haem groups has a great influence on the spreading properties of haemoglobin.

Do these changes in the spreading properties only occur when oxygen is added or removed, or can the oxidation of Fe^{++} to Fe^{+++} , as in oxidation of reduced cytochrome, also influence the spreading properties?

Cytochrome forms monolayers on solutions of a wide range of pH [Harkins & Anderson, 1938] (see Fig. 3). At pH 9.6 (close to the isoelectric point of cytochrome) a monolayer is easily formed and the maximum spreading is

reached after 5 min. At lower pressure the surface pressure curve is remarkably flat. When hydrosulphite is added, the unfolding time becomes greater; after 5 min. the area is only 25 % of the maximum area and the maximum is not reached, probably because a certain amount of the cytochrome is dissolved in the solution in the trough.

When the experiment is repeated, after the hydrosulphite has been oxidized by the air, the normal spreading of oxidized cytochrome is found again. Here also a difference in spreading properties between the oxidized and reduced forms can be found. The differences are just the opposite of those between oxygenated and reduced haemoglobin: oxyhaemoglobin forms monolayers with greater difficulty than reduced haemoglobin, oxidized cytochrome more easily than reduced cytochrome.

SUMMARY

The maximum areas of all haemoglobins in the compressed state are the same, 9400 sq. cm. per mg. This indicates that they are built up from the same number of amino-acids per Svedberg unit.

The times after which the maximum area is reached at the isoelectric point are different for the haemoglobins of different species.

This time is independent of the total ion concentration of the solution on which the films are spread, provided that the *pH* is that of the isoelectric point of the protein.

Small concentrations of urea have hardly any influence on the spreading properties of haemoglobin.

On concentrated solutions of urea the unfolding time is shorter, and the surface pressure curve is flatter, but there are no differences in the behaviours of horse and sheep haemoglobins, although horse haemoglobin is dissociated into molecules of half the normal molecular weight and sheep haemoglobin is not.

There are differences in unfolding time between oxygenated and reduced haemoglobin. Reduced haemoglobin spreads more rapidly than oxyhaemoglobin, but oxidized cytochrome spreads more rapidly than reduced.

Muscle haemoglobin does not form monolayers on solutions of a *pH* between 4 and 11, but reduced muscle haemoglobin forms monolayers at *pH* 6.8. The maximum area is reached after 5 min.

I wish to express my deep gratitude to Prof. D. Keilin for his help and interest in this work and to Prof. N. K. Adam for reading the manuscript.

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CCXVIII. SOME OBSERVATIONS ON PEPTIC DIGESTION OF EGG ALBUMIN

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(Received 7 August 1939)

With the classical methods of investigating the enzymic hydrolysis of proteins much information has been obtained about the specificity and mode of the enzymes. By studying the split products one should also be able to gain some knowledge about the structure of the proteins themselves. For this purpose much work has been done on the isolation and identification of the end-products of complete digestion. But it would also be of great importance to find out what happens during the earlier stages of the digestion. Some investigations along these lines have previously been carried out in this laboratory. Svedberg & Eriksson [1934] and Annetts [1936] have studied the digestion products formed by the action of papain on egg albumin. In this paper some results will be given from work with pepsin on the same protein.

The egg albumin was crystallized according to the method of Hopkins [1900] modified by La Rosa [1927]. It has been found, however, by means of the improved electrophoresis methods, that this material generally contains a component with slightly lower mobility than the remainder. In four out of five preparations investigated, this component has been present to some extent. As comparative determinations have always been made this should not influence the results, but it somewhat complicates the work. On the whole, egg albumin does not seem to be such a simple and "typical" protein as is generally claimed in the literature.

The enzyme used was Northrop's crystalline pepsin. The digestion was usually carried out in 0.5 or 1*M* acetic acid at 35–40° with a pepsin concentration of 0.06 %. The degree of hydrolysis was determined by the Linderström-Lang acetone titration method.

The partially digested solution was first investigated in the electrophoresis apparatus, using the *Schlieren* method [Tiselius, 1937]. The usual method of dialysing the solution to be investigated against the electrolyte solution used as a supernatant in the electrophoresis tube cannot be applied in this case, as no doubt some low molecular weight material would dialyse out of the bag. Instead the acetic acid-digestion mixture was investigated directly, with an acetic acid solution of the same concentration as a supernatant. This deviates from the common practice of using buffered electrolyte solutions as media for protein electrophoresis experiments and makes accurate measurements of mobilities impossible. Under these conditions observations on the descending boundary (the ascending boundary is unstable) are, however, particularly well suited to give information regarding the homogeneity of the substances in the sample investigated, as the diffusion is counteracted by the automatic sharpening of

the boundaries, which therefore become exceptionally well developed. The low conductivity of the solution also permits the use of high voltage, which gives a rapid separation of the components. This procedure applied to a solution of undigested egg albumin gives one sharp boundary, which however after prolonged electrophoresis splits up into two, migrating close to each other (see above). The digestion mixtures contained one more well developed boundary of much lower mobility (see Fig. 1) evidently representing a group of substances with electrochemical properties distinctly different from those of undigested egg albumin. It is particularly interesting that in no case were any boundaries intermediate between these two observed: increasing time of digestion only changes the relative proportions between the two components. Evidently these experiments suggest that the change brought about by the digestion is a rather abrupt one, which does not give rise to any stable intermediate products, at least not as far as electrochemical properties are concerned.

The slow boundary, after prolonged electrophoresis with compensation in the large type apparatus [Tiselius, 1938], splits up into at least three different components with closely similar mobilities. So far we have not made any attempts to separate these but have only attempted to characterize them as a group of split products in comparison with the undigested egg albumin.

The slow fraction is most easily isolated by electrophoretic separation in the large apparatus, the fast fraction (undigested protein) by dialysis. The electrophoretic isolation of the latter in the acetic acid medium is difficult on account of the blurring of the ascending boundaries.

Investigation of the high molecular weight fraction

The acid digestion mixture was neutralized with NaOH. The acid itself denatures the protein partly so that by the neutralization a precipitate is formed in a *pH* region around the isoelectric point. The *pH* was brought to 8, where the protein was soluble and the solution obtained was only faintly opalescent. This was then dialysed in cellophane bags to get rid of the low-molecular split products. The following four solutions were compared:

A. Protein dissolved in phosphate buffer of *pH* 8.

B. Protein treated with acetic acid and then brought to *pH* 8.

C. Protein digested with pepsin in acetic acid 1 hr. and then brought to *pH* 8. Dialysed in cellophane bag. The acetone titration shows an increase in amino-groups corresponding to 0.54 ml. 0.1*N* HCl per 0.178 g. protein. 70 % of the total nitrogen is retained by the membrane in the dialysis.

D. Protein digested with pepsin 10 hr., brought to *pH* 8, dialysed. Increase of amino-groups, 0.99 ml. 0.1*N* HCl per 0.178 g. protein. 50 % of the total nitrogen retained by the cellophane.

Electrophoresis. The electrophoretic mobility measurements were carried out in



Fig. 1. Electrophoresis of egg albumin before *A* and after *B* digestion with pepsin. Potential gradient 18 V./cm. Time interval between exposures 15 min.

phosphate buffer of ionic strength 0.1 and *pH* 8.0. The following results were obtained:

Solution	Mobility*
A	7.12
B	7.06
C	7.18
D	6.85

* Throughout this paper electrophoretic mobilities are expressed in units of 10^{-5} , sedimentation constants in units of 10^{-13} and diffusion constants in units of 10^{-7} .

The values found are in good agreement within the limits of experimental error.

Sedimentation velocity. The sedimentation constants were determined in the ultracentrifuge using a centrifugal field of 350,000 times gravity. The solutions were made 0.2*M* in NaCl to eliminate the Donnan effect. The following sedimentation constants were found:

Solution	Sed. const.
A	3.68
B	3.69
C	3.67
D	3.64

The shapes of the sedimentation curves were very much alike and did not indicate any difference in the compositions of the four solutions.

Diffusion. The measurements were made on the same solutions as the sedimentation velocity runs. Solution B gave the diffusion constant 7.8, which is the normal one for egg albumin. For solution D the value 7.13 was obtained. The agreement is not very good. The values from different parts of the curves indicate that the solution D contains a certain amount of aggregated material. The sedimentation curves, which are better than diffusion curves in detecting non-homogeneity, show that this amount must be very slight.

Low-molecular weight fraction

The low-molecular weight component has a much lower electrophoretic mobility than the other one (see Fig. 1). The split product was isolated after different digestion times. To get some idea of its dispersity and molecular size, diffusion constant determinations were made.

Digestion time in hr.	Increase of NH_3 in ml. 0.1 <i>N</i> HCl per 0.1 g. prot.	Diff. const.
3	0.79	33.7
44	1.84	32.6
63	2.11	34.5

The values of the diffusion constants for the materials isolated at different degrees of digestion agree within the limits of experimental error. They also seem to have the same mobility. Thus the same split product seems to be formed during the whole course of the digestion. It can, however, only be very incompletely characterized with these methods.

In order to get an estimate of its molecular weight a sedimentation equilibrium run was made in the oil turbine ultracentrifuge. The scale method of observation was used. The centrifuge was run at a speed of 55,000 r.p.m. The height of column of solution was 0.48 cm. and the temperature in the cell 28.4°. The equilibrium seemed to be established in 30–35 hr. The exposures used

for calculation were taken after 43, 45 and 47 hr. The values of the molecular weight obtained at different distances from the centre of rotation showed a drift from the top to the bottom of the cell, showing that the material is not homogeneous with regard to molecular weight. The weight average molecular weight was calculated by means of a formula worked out by O. Quensel (unpubl.)

$$\text{mol. wt.} = K \frac{z}{pc_0 X},$$

where

z = scale line displacement,
 p = number of intervals used,
 c_0 = initial concentration,
 X = mean distance from centre of rotation,

and

$$K = \frac{RT}{\omega^2 (1 - \bar{V} \rho) G a b},$$

where

R = gas constant,
 T = absolute temperature,
 ω = angular velocity,
 \bar{V} = partial specific volume,
 ρ = density of solvent,
 G = enlargement factor,
 a = thickness of column of solution,
 b = scale distance.

The value obtained in this way was from three different exposures 1080, 1082, and 1065, which gives 1080 as a mean value for the weight average molecular weight of the digestion product.

DISCUSSION

In the aforementioned investigation of the action of papain on egg albumin it was found that the whole material underwent a slight preliminary change before it was broken down to small particles. The molecular weight was not changed, but the sedimentation constant decreased, indicating a change of shape. The electrophoretic curves showed no resemblance with those of the undigested protein. In the case of pepsin it seems that the enzyme attacks one after the other of the protein molecules and immediately breaks them down to very low-molecular weight split products. The amount of these increases as the digestion proceeds. These products have an average molecular weight of about 1000. The mean molecular weight of the amino-acid residues in egg albumin is 124, which gives eight as the average number contained in the peptides formed. The split product is naturally not homogeneous, for smaller and larger peptides may also be formed. The part of the protein which has not been broken down to small particles seems to be almost unchanged egg albumin if only the properties here investigated are considered. The electrophoretic mobility and the size and shape of the molecules are nearly the same as in the native egg albumin. Unlike the split products it gives a precipitate with anti-egg albumin rabbit serum. Part of the material becomes insoluble at the isoelectric point, but this denaturation is due to the effect of the acetic acid and not of the enzyme.

One may assume two distinctly different types of mechanism for the enzymic splitting of large molecules of which the two extremes would be: (1) all molecules are simultaneously but gradually broken down to products which are no longer attacked by the enzyme, (2) only a few molecules are attacked in each time interval but these are quite rapidly broken down to the end-products. In the former case a digestion mixture should contain a number

of products which would show a more or less continuous variation in size and other properties between those of the original and the end-products; in the latter case the mixture should contain unchanged large molecules and fully digested end-products, but no appreciable amount of intermediate substances. The results obtained in the present investigation give strong evidence for the second alternative in the case of digestion of acid-denatured egg albumin with crystalline pepsin.

It should be observed that the denaturation of proteins has been described as an "all or none" reaction of a similar character [Pedersen, 1931; Anson, 1938].

SUMMARY

A study of solutions of egg albumin partially digested with crystalline pepsin in acetic acid medium, using electrophoresis, sedimentation and diffusion methods, indicates that appreciable amounts of unchanged, acid-denatured egg albumin are present even after prolonged digestion, together with the end-products, whereas no marked amount of intermediary split products could be observed. This result suggests that the peptic digestion of egg albumin is more similar to the "all or none" type of reaction than a gradual breakdown process.

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CCXIX. THE FATE OF OXALOACETIC ACID IN DIFFERENT ORGANS

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SZENT-GYÖRGYI and collaborators [1935] have shown that oxaloacetic acid (in this paper referred to as OAA) plays an essential part in the system of C_4 -dicarboxylic acids concerned with the transport of H from the donators to O_2 . OAA, by accepting H, is reversibly hydrogenated to *l*-malic acid (very likely by the yellow enzyme of Warburg) [Banga, 1937, 1, 2; Laki, 1937], and dehydrogenated back to OAA by malic dehydrogenase.

Apart from the hydrogenation to *l*-malic acid, the aqueous solution of OAA spontaneously decomposes slowly into pyruvic acid (referred to as PUA in this paper) and CO_2 , and in tissues it undergoes several other reactions. Decarboxylation of OAA is increased in presence of either surviving or heated tissue. Further, OAA and glutamic acid undergo rapid conversion in the tissue into α -ketoglutaric acid and aspartic acid [Szent-Györgyi *et al.* 1935; Braunstein & Kritzmann, 1937]. Knoop & Martius [1936] proposed a theory of the probable formation of citric acid in tissues from OAA and a substance containing three carbon atoms. A year later Orten & Smith [1937] discovered that in fact the injection of large amounts of C_4 -dicarboxylic acids brought about an increased excretion of citric acid in urine. This citric acid must have been formed as an intermediary product of the C_4 -dicarboxylic acids. On this Krebs & Johnson [1937] based a theory of the breakdown of trioses, thus combining in a cycle the system of C_4 -dicarboxylic acids of Szent-Györgyi with the reactions of formation and breakdown of citric acid according to Knoop & Martius. In my recent work on pigeon muscle [1937, 1] I was unable to confirm the formation of citric acid from OAA, and therefore I could not accept the citric acid cycle as a mechanism of metabolism in the muscle.

As in these fundamental questions there are two opposite views which need to be elucidated, it seemed advisable to make quantitative investigations to decide if the sum of the hitherto known breakdown products of the OAA corresponds within the limit of error to the added amount of OAA: it could then be concluded that other reactions and products of metabolism than those investigated could only play a secondary part.

For this purpose I have quantitatively investigated the fate of OAA in many tissues of warm-blooded animals by estimation of the following products originating from OAA: PUA, lactic acid, citric acid, *l*-malic acid.

In order to account for that part of the lactic acid which is formed from PUA, I was obliged first to examine the spontaneous formation and destruction of lactic acid in the various tissues. Experiments to determine the total amount of carbohydrate which might have been resynthesized from lactic acid did not yield any results, as no relation could be found between the total carbohydrate and the addition of OAA.

The total balance (of PUA, *l*-malic acid, lactic acid and, in kidney, also citric acid) gave in kidney, lung, spleen, placenta and peripheral nerves a practically complete recovery of added OAA. In these organs OAA and the above-mentioned substances, under my conditions, virtually do not undergo any other important reactions than those referred to.

In muscle (pigeon, cat) I found a residue of only 70 %, in liver 50 % and in brain 20 %, calculated as the sum of the above-mentioned substances. It seems that in these organs the loss is due to other changes and reactions of formed PUA, leaving dismutation according to Krebs or total combustion of the formed lactic acid as alternatives.

Following these balance investigations I investigated the various enzymes taking part in the metabolism of OAA. In the first place the enzymic hydrogenation of OAA to *l*-malic acid, secondly the enzymic condensation of OAA and PUA to citric acid in the kidney, thirdly, the transamination of glutamic acid with OAA and, finally, the decarboxylation of OAA to PUA were investigated.

EXPERIMENTAL

All experiments were performed on the organs of cats and pigeons; the animals were killed by a blow on the head and subsequently bled. The organs were instantly taken out, minced in the Latapie mill, weighed and the minced tissue instantly transferred into the already prepared solutions.

Every investigation was started 3–5 min. after the death of the animal except in the case of peripheral nerve, the separation of which (2.5 g. per animal) took about 15 min. Because of the insufficient quantity of nerve tissue obtained after passage through the Latapie mill, I had to resort to mincing the nerves with a pair of fine scissors.

As I found that the same organs of different animals showed metabolic deviations up to 100 %, all balance investigations were always carried through in one experiment. In those cases where the weight of one organ proved to be insufficient, another or several others of the same kind were used, care being taken to kill the animals at the same time and to mix the minced tissues thoroughly.

As preliminary experiments, to be described later, had shown the following conditions to give the most satisfactory results, these conditions were maintained: 5 g. minced tissue, suspended in 30 ml. Ringer-phosphate solution [Banga & Szent-Györgyi, 1937] containing 50 mg. OAA (neutralized in not more than 0.5 % solution), were shaken in 200 ml. Erlenmeyer flasks under aerobic conditions, the temperature of the thermostat being kept at 38°. Small glass funnels prevented evaporation.

After 2 hr. shaking, the precipitation of proteins was carried through with trichloroacetic acid for the estimation of citric acid, while for all other substances tungstate was used. Quantitative tests for OAA, PUA, *l*-malic acid, lactic acid and citric acid were then performed.

The analytical methods used were the following: OAA, PUA and *l*-malic acid were determined according to Szent-Györgyi & Straub [Szent-Györgyi *et al.* 1935], lactic acid according to Friedemann *et al.* [1927] in the apparatus of Fuchs [1933], citric acid gravimetrically after Cometiani [1931], as described by Breusch [1937, 1]; in the present study a modification of the latter method was used, in which the precipitate of pentabromoacetone after filtration on a glass filter was dried in a non-evacuated desiccator over P_2O_5 at room temperature.

The OAA used was synthesized according to Wohl & Österlin [1901] and melted at 150–152°.

To allow for the absorption by precipitated protein of the estimated substances, a correction of 20 %, added to the found values, was made according to the findings of Straub [cf. Szent-Györgyi *et al.* 1936]. Repeating these investigations, I found that the loss due to adsorption was also 20 % in the case of the dilutions used here, with deviations of ± 5 %.

In order to find out whether lactic acid was formed at all after addition of OAA it was necessary to test the formation of lactic acid with and without OAA. Furthermore, it had to be tested whether and to what extent lactic acid added to tissue was oxidized.

The results are shown in Tables I and II.

Table I. *Tissue incubated without addition of lactic acid*

Organ	Found mg. lactic acid	
	Initial	After 2 hr.
Muscle, cat	18	32
Muscle, pigeon	27	45
Liver	5	8.8
Kidney	6	4.4
Spleen	8	10
Lung	7	8
Placenta	6	11.4
Brain	11	9.7
Nerve (periph.)	5	3.3
Pancreas	5	5

Table II. *Tissue incubated with addition of 30 mg. lactic acid*

Organ	Found mg. lactic acid	
	Initial	After 2 hr.
Muscle, cat	44	60
Muscle, pigeon	56	74
Liver	34	32
Kidney	31	33
Spleen	34	37
Lung	33	33
Placenta	31	32
Brain	34	31
Nerve (periph.)	33	34
Pancreas	—	—

No organ except muscle shows a considerable spontaneous increase of lactic acid. Little added lactic acid disappears from any organ. Special experiments showed that addition of fumaric acid (which originates from OAA in the metabolism of some tissues) does not change the balance of lactic acid to any appreciable degree. Within the limit of error under these conditions the values are the same with and without addition of fumaric acid.

In Table III column 2 shows the amount of OAA still present after 2 hr. incubation, column 3 the formed fumaric+*l*-malic acid; column 4 the citric acid; column 5 the PUA formed. Column 6 contains the total lactic acid; column 7 the lactic acid formed without addition of OAA (see Table I); column 8 the lactic acid formed from OAA, and column 9 the sum of all products found, calculated as OAA. Column 10 gives the percentage of OAA recovered from added OAA.

According to the rapid equilibrium between *l*-malic and fumaric acid in the presence of fumarase in every tissue [Breusch, 1937, 2] the equivalent of fumaric

Table III. *Quantities (mg.) present after 2 hr. incubation*

1	2	3	4	5	6	7	8	9	10
Organ	OAA	Fumaric + <i>l</i> -malic acid	Citric acid	PUA	Total lactic acid	Lactic acid Table I	Lactic acid from OAA	Sum as OAA	% recovery OAA
Liver	0	17.1	0	2.4	12.6	8.8	3.8	26.3	52.6
Kidney	0	25.3	10	1.0	8	4.4	3.6	44.0	88.0
Muscle, pigeon	0	15.2	0	3	56	45	11	35.5	71.0
Muscle, cat	0	6.7	0	10	42	32	10	36.6	73.2
Brain	0	5.2	0	3.5	8.5	9.7	0	10.3	20.6
Pancreas	0	6.5	0	20.0	7	5	2	39.3	78.6
Lung	0	0	0	28	10	8	2	45.0	90.0
Spleen	0	0	0	31	12.2	10	2.2	48.8	97.6
Placenta	0	0	0	27.6	12.4	11.2	1.2	42.4	84.8
Nerve (periph.)	0	0	0	28.3	5.4	3.3	2.1	45.6	91.2

acid formed was added to the value of *l*-malic acid. The same procedure was used in the case of citric acid, corresponding to the equilibrium ratio of the enzyme aconitase [Martius, 1938], citric acid/*isocitric* acid = 9/1.

The result of these investigations is as follows. All organs accelerated the decarboxylation of OAA. In all tissues OAA completely disappeared after 2 hr.

Only five of the organs investigated, i.e. muscle, liver, kidney, pancreas and brain, are able to hydrogenate OAA to *l*-malic acid. In these five organs only, is this step of redoxidation of the C₄-dicarboxylic system used for the transport of H. In brain I did not always find a reduction of OAA; some brains used immediately after death did not show a measurable formation of *l*-malic acid.

Thus the relation is revealed, that a tissue either has or has not at all the ability to reduce OAA and PUA. This result establishes connexion with results of Green *et al.* [1937], who found that the oxidoreduction between PUA and OAA on the one hand, and C₃-compounds on the other, is brought about by the same group of mutases.

The brain stands midway between these two groups of organs; it combines a poor capacity for reducing OAA with a great ability to metabolize PUA [Peters, 1936; Peters & McGowan, 1937; Long, 1938; Barron & Lyman, 1939; Annau & Mahr, 1937; Weil-Malherbe, 1937; McGowan, 1937; Elliot *et al.* 1937; etc.]; in this case only a very small part of the PUA disappearing is recovered as lactic acid. PUA also disappeared in liver and kidney without giving rise to detectable amounts of lactic acid [cf. Deutike & Jens, 1938; Annau & Mahr, 1937].

In confirmation of my previous work [Breusch, 1937, 1] I could find no ability to synthesize citric acid in any kind of muscle; namely breast and stomach muscle of pigeons and skeletal and heart muscle of cats. It was only in kidney that after incubation with OAA I observed formation of citric acid. After addition of larger amounts of OAA and PUA together and incubating under anaerobic conditions in some organs (liver, brain and lung) a small amount of citric acid could be detected. This will be discussed in the section on citric acid formation. Even under these most favourable conditions I did not find any citric acid formation in any of the muscles examined.

As can be seen from column 9 of Table III, within the limit of analytical error, all metabolites of OAA originating from the metabolism of the following organs can be recovered: kidney, lung, placenta, spleen and peripheral nerves, giving thus a practically quantitative balance of the decomposition of OAA. Other reactions of OAA which may be found in future can be regarded as of minor importance in these organs.

No such quantitative result is revealed by the analysis in brain (loss of 80 %), in liver (loss of 50 %) and in muscle (loss of 30 %). In these organs a part of the PUA, or of the lactic acid formed from the latter, seems to be totally burned or to be transformed into other products.

The reduction of OAA to l-malic acid

Banga [1937, 1] was the first to try to isolate the complex of enzymes transporting the H from the donator to OAA. By extracting the fresh minced muscle with ice-cold water, centrifuging the extract, and precipitating with ice-cold acetone she succeeded in isolating this complex and found it to be highly sensitive. A somewhat modified method was employed by Green *et al.* [1937].

I found that the system responsible for reduction of OAA is nearly quantitatively extracted by ice-cold alkaline phosphate solution and that it can be precipitated by saturating with ammonium sulphate.

The method is as follows: immediately after the death of the animal 50 g. pigeon muscle are minced in an ice-cold Latapie mill, the minced tissue ground in an ice-cold mortar with 200 ml. ice-cold 1 % Na_2HPO_4 for 10 min. and then pressed through a cotton cloth. The impure filtrate is centrifuged for 10 min. in ice-cold tubes, and the supernatant then filtered through cotton-wool. The almost clear filtrate is saturated with crystallized ammonium sulphate and the precipitated protein filtered in the ice-box through two filter papers (3 hr.). After one washing with sat. ammonium sulphate the filter with the wet precipitate is dried by squeezing it out between filter papers. The whole precipitate is dissolved in Ringer-phosphate solution and freed from the small insoluble residue by filtration.

This solution is stable in the ice-box for about 6–10 hr. It is virtually free from donators; and incubation with OAA alone does not yield *l*-malic acid. If the protein precipitate is not washed with sat. ammonium sulphate it still contains sufficient coenzyme; but after it has been washed, coenzyme must be added in order to obtain reduction to *l*-malic acid after addition of a donator.

Banga [1937, 1] found hexosediphosphate and monophosphate, Green *et al.* [1937] triosephosphate to be effective H donators, as well as glycerophosphate. I can confirm this, but in my enzyme preparation glycerophosphate was not dehydrogenated. Further, I found phosphoglyceric acid and, to a small degree, dihydroxyacetone to be effective donators. Glucose, fructose, lactic acid, phospholactic acid, PUA, glycerol, glycerophosphate (α - and β -), β -hydroxybutyric acid, isocitric acid, α -ketoglutaric acid and α -hydroxyglutaric acid did not show any donator activity; *dl*-glyceraldehyde even inhibited.

The pH optimum of OAA-reduction I found to be in the range of pH 8–8.5. For examining the influence of dilution with Ringer-phosphate solution on the minced tissue suspension the conditions given in Table IV were used.

Table IV

10 g. of minced breast muscle of pigeons and 100 mg. neutralized OAA in 10 ml. Ringer-phosphate solution were shaken at 38° for 1 hr. under aerobic conditions; the dilution by further addition of Ringer-phosphate can be seen below.

Dilution	× 2	× 3	× 4	× 5	× 6	× 7	× 8
Found <i>l</i> -malic acid (mg.)	10	16	19	18	15	13	7

The amount of *l*-malic acid formed is practically unaffected by as much as 7-fold dilution.

The influence of the quantity of added OAA on the formation of *l*-malic acid is shown in Table V.

Table V

Conditions: 5 g. minced kidney tissues of cats; addition of 5, 10, 15, 20, 30, 50 mg. neutralized OAA in 20 ml. Ringer-phosphate solution; 1 hr. anaerobically with shaking; at 38°.

Addition mg. OAA	5	10	15	20	30	50
Fumaric + <i>l</i> -malic acid formed (mg.)	4.3	8.0	10.5	11.0	17.0	25.3
As % of added OAA	86	80	70	55	57	50

With increasing concentration the percentage amount of fumaric + *l*-malic acid formed decreases.

As already shown by Szent-Györgyi and collaborators, the reduction of OAA is a reaction of the zero type; i.e. even minimum concentrations of OAA are metabolized.

As, under physiological conditions, the concentration of OAA is very low (it cannot be detected and must, therefore, be lower than 1 mg. per 100g.), practically

all is reduced; only by adding amounts of OAA far in excess of the physiological limits do decarboxylation to PUA and, in kidney, condensation to citric acid, come into action.

Citric acid formation from OAA

Since I could not confirm the establishment of the citric acid cycle of Krebs & Johnson [1937] as a main step in tissue metabolism [Breusch, 1937, 1], it seemed important to undertake a closer investigation into the citric acid formation in tissues.

As can be seen in Table III, it is only minced kidney which, incubated with OAA alone, can form citric acid; while anaerobically in the presence of larger amounts of OAA and PUA, liver, brain and lung also form small amounts of citric acid. Muscle under the same conditions did not yield any analysable amount of citric acid.

According to the theory of Knoop citric acid is formed by condensation of OAA and PUA and subsequent oxidation of one C-atom. I therefore investigated which C_3 - or C_2 -compounds when incubated together with OAA and kidney tissue gave a higher value for citric acid than OAA alone. Among all the compounds tried, I only found PUA, glyceric acid, dihydroxyacetone and, to a very small degree, phosphoglyceric acid to be effective (Table VI).

Table VI

The conditions used were as follows: to 5 g. minced kidney tissue and 15 ml. Ringer-phosphate are added 100 mg. OAA and 50 mg. of the acid to be tested, neutralized in 15 ml. Ringer-phosphate solution. With 100 mg. OAA alone and minced kidney tissue I found in 2 hr., aerobically, 12 mg. citric acid; the Table shows the increase of citric acid formation due to the addition of the various substances:

Additions	Increase of citric acid formation %	Additions	Increase of citric acid formation %
Glycerophosphate, α - and β -	0	Glyceric aldehyde	Inhibition
Lactic acid	0	β -Hydroxybutyric acid	0
Phospholactic acid	0	Glyceric acid	100
Acetic acid	0	Pyruvic acid	200
Acetaldehyde	0	Dihydroxyacetone	100
Ethanol	0	Phosphoglyceric acid	50
Glycolaldehyde	0	Glycerol	0

These results confirm the theory of Knoop & Martius [1936], according to which citric acid formation from OAA and PUA happens not only *in vitro* but also in the organism. Dihydroxyacetone, glyceric acid and, to a lesser extent, phosphoglyceric acid, also have this ability: whether directly or after transformation into PUA remains unsolved.

No other acid can replace OAA in the formation of citric acid. Under the conditions of my experiments all the substances examined without addition of OAA were unable to form citric acid: succinic acid, fumaric acid, *l*-malic and *dl*-malic acid, β -hydroxybutyric acid, butyric acid, maleic acid, glutamic acid, α -ketoglutaric acid and all substances with 2 or 3 C-atoms known to be normally present in tissue.

As I did not find any of the C_4 -dicarboxylic acids other than OAA effective, it seems certain that, in the experiments of Orten & Smith [1937] concerning the transformation of injected C_4 -dicarboxylic acids into citric acid excretable in the urine, these reactions must have passed through the stage of transformation into OAA.

The same applies to the investigations of Simola [1938], who found that injected β -hydroxybutyric acid causes an increased citric acid excretion in urine. In a paper of Kühnau [1928], which has unfortunately aroused insufficient attention, this author has shown that liver partly transforms β -hydroxybutyric acid into C_4 -dicarboxylic acids.

The failure to obtain citric acid by incubating kidney with fumaric + *l*-malic acids is probably due to the fact that under my conditions the capacity for re-oxidizing *l*-malic acid into OAA is slower than the reaction leading to the formation of *l*-malic acid from OAA. Thus a sufficiently high concentration of OAA was never reached to yield citric acid.

The possibility of isolating a substance from tissue depends on the ratio of enzymic velocities. A substance will accumulate if the synthesizing enzymes act more rapidly than the catabolizing ones. This may be the case in muscle, where, in contradiction to the citric acid cycle of Krebs, I could not detect citric acid formation; here citric acid is perhaps more quickly broken down than formed. I therefore investigated the ability of different organs to consume citric acid (Table VII).

Table VII

Conditions: 5 g. minced tissue and 30 mg. citric acid in 30 ml. Ringer-phosphate solution; (a) immediately analysed, (b) after 2 hr. aerobic incubation with shaking; at 38°.

Organ	Immediately analysed mg. citric acid	After 2 hr. incubation mg. citric acid	Disappearance %
Liver	26	0	100
Muscle, cat	25	21	16
Kidney	26	12	58
Brain	27	22	19
Lung	27	23	16
Spleen	25	22	12

In all the organs 1/6 to 1/7 of added citric acid disappears; this is [Breusch, 1937, 1] due to a balance between citric and isocitric acids. This was confirmed by Martius [1939], who by exact analysis with plant aconitase found the equilibrium to be at 10% isocitric acid and 90% citric acid. The enzyme "aconitase" is not identical with fumarase [Breusch, unpublished; Martius, 1938; Johnson, 1939; da Cunha & Jacobsohn, 1939]. Johnson found the balance to be at 80% citric acid, 16% isocitric acid and 4% aconitic acid; a value better corresponding to those obtained by myself than to those of Martius.

It is only in liver and kidney, that citric acid disappears to a far greater amount than that corresponding to this equilibrium.

Though muscle proves in the Thunberg test to have a great ability to dehydrogenate isocitric acid, it is not able to break down citric acid to an appreciable extent.

In pigeon muscle the breakdown of citric acid is variable and seems to be dependent on the season; I found that in summer about 20%, in winter 30–40%, citric acid disappeared.

In confirmation of these results Martensson [1938] showed in perfusion experiments, using the cat's hind leg, that in the living muscle also, citric acid practically does not disappear from the perfusion fluid; on the contrary small amounts (of the order of μ g.) are formed. Contrary to the behaviour of muscle, in the perfused liver and kidney large quantities of citric acid disappear, thus exactly corresponding to my results.

As has been proved by two completely different methods, muscle neither

forms nor breaks down appreciable amounts of citric acid. Therefore the inability to find citric acid in muscle after incubation with OAA cannot be due to a quick combustion of citric acid first formed.

To inhibit the combustion of citric acid, perhaps already formed, the experiments were repeated under the most favourable anaerobic conditions adding OAA and PUA to various tissues. Condensation of OAA with PUA into citric acid occurs in kidney tissue anaerobically also; the removal of the extra C-atom, formed during condensation, seems to be brought about either by dismutation with a keto-acid according to Krebs; or the condensation product is also oxidized to pentabromacetone during analysis and thus estimated as citric acid.

Under these conditions different organs show a small ability to condense and forming citric acid. Even under these most favourable conditions all kinds of muscle show no citric acid formation.

Table VIII

Conditions: 5 g. fresh minced organ were put with 15 ml. Ringer-phosphate solution into a cylindrical evacuable vessel, fitted with a ground end and stopcock. In a small inner tube, standing in the large vessel, were placed 100 mg. OAA and 50 mg. PUA, precisely and separately neutralized to litmus, in 15 ml. Ringer-phosphate. The whole was closed and evacuated for 3 min. with a motor-oil pump. Then the stopcock was closed, the acids mixed *in vacuo* with the tissue suspension by turning and the whole anaerobically incubated for 1 hr. at 38° and then instantly deproteinized with trichloroacetic acid. A control with the acids and Ringer-phosphate solution alone was treated under the same conditions.

The different organs yielded the following synthesis:

Organ	Kidney	Liver	Pigeon muscle	Cat muscle	Lung	Brain	Spleen	Placenta	Intestine
mg. citric acid formed	28	2	0	0	1	1	0	0	0

Table VIII shows that, even under these most favourable conditions, only kidney synthesizes citric acid to a large extent; liver, brain and lung do so to a small extent.

To see which of the competing reactions of OAA, the reduction to *l*-malic acid or the condensation to citric acid, has the advantage in kidney tissue, I investigated (Table IX) the dependence on the concentration of added OAA of the formation of *l*-malic acid and citric acid.

Table IX

Conditions: 5 g. minced kidney and varying amounts of OAA, neutralized in 30 ml. Ringer-phosphate solution, shaken 1 hr. anaerobically at 38°.

Added amount of OAA mg.	Found <i>l</i> -malic + fumaric acid mg.	Found citric acid mg.
5	4.3	0
10	9.0	0
15	10.5	0
20	12.0	0
30	17	1
50	29	2
100	40	12
200	45	27

Under physiological conditions the reduction of OAA to *l*-malic acid happens practically quantitatively, as already shown by Szent-Györgyi & Banga. Only with concentrations of OAA far beyond the physiological content of tissue is

citric acid formed in kidney by condensation of OAA and the PUA which is formed from it.

For metabolism, that is the transport of H from trioses to cytochrome, it seems that only the reduction of OAA to *l*-malic acid and not the formation of citric acid is important, the latter according to my results only happening in the presence of a surplus of OAA.

The formation of citric acid in kidney seems to be a means by which the organism is able to get rid of a surplus of the C₄-dicarboxylic acids and to excrete them; since the C₄-dicarboxylic acids are not themselves excretable in the urine [Flaschenträger & Bernhard, 1929; 1937; Balassa, 1937]. Citric acid is a constant constituent of urine; everyone excretes 100–300 mg. per day [Östberg, 1931; Fürth *et al.* 1934; Sherman *et al.* 1936].

The formation of citric acid in the kidney appears to be a mechanism by which the level of C₄-dicarboxylic acids in the tissues is kept constant, since these C₄-dicarboxylic acids, according to our increasing knowledge, are the most important carriers of H from the trioses to cytochrome. Furthermore, the formation of citric acid in kidney seems to be a mechanism by which the organism synthesizes α -ketoglutaric acid (a breakdown product of citric acid) which, according to Braunstein & Kritzmann [1937], plays an important part as carrier of NH₃ in the formation and breakdown of amino-acids.

By these experiments the results of Orten & Smith [1937], who found an increase of citric acid excretion after injection of the various C₄-dicarboxylic acids, are elucidated as a mechanism for the removal of surplus C₄-dicarboxylic acids.

I have attempted to determine the properties of the enzyme forming citric acid in the kidney. The *pH* optimum is at the neutral point. The dependence of citric acid formation on dilution is shown by the following experiment.

Table X

Conditions: 5 g. minced kidney and 100 mg. neutralized OAA in 5, 10, 20, 40 and 100 ml. Ringer-phosphate solution were shaken aerobically for 1 hr. at 38°.

Ringer-phosphate (ml.)	5	10	20	40	100
Dilution of tissue	$\times 2$	$\times 3$	$\times 5$	$\times 9$	$\times 21$
Citric acid formed (mg.)	19	18	19	20	10

The amount of citric acid formed under the conditions of the experiment is independent of dilution up to a $\times 9$ dilution.

The citric acid formation is not inhibited by *M*/15 NaF or by *M*/1000 HCN. *M*/2000 arsenious acid inhibits it 50 %, *M*/750, 70 %. *M*/100 HCN and *M*/750 iodoacetate inhibit 100 %.

It is very difficult to bring the condensation system into solution. Neither alkaline, neutral, nor acid phosphate solutions are effective. The enzyme is destroyed by heating in the water-bath. I propose to call this enzyme "citro-genase".

Condensation of OAA with glutamic acid

Added OAA disappears very quickly from tissue after addition of *l*(+) glutamic acid, as found by Szent-Györgyi & Banga. It is very likely that this reaction occurs by the transaminating enzyme of Braunstein & Kritzmann [1937, 1939], yielding aspartic and α -ketoglutaric acids from OAA and glutamic acid. I investigated (Table XI) the capacity of some organs to catalyse this reaction.

Table XI

Conditions: To 2 g. minced organ in 5 ml. Ringer-phosphate 25 mg. OAA and 50 mg. *l*(+)-glutamic acid, neutralized in 10 ml. Ringer-phosphate were added; the mixture was incubated for exactly 10 min. at 38°, then deproteinized with tungstate and OAA determined colorimetrically. In control experiments without glutamic acid decarboxylation and reduction of OAA were determined and subtracted.

The following Table shows how many mg. of OAA are metabolized under these conditions in 10 min. by 1 g. wet tissue.

Cat muscle	Liver	Kidney	Lung	Cat embryo muscle	Brain	Washed erythrocytes
7 mg.	5 mg.	8 mg.	8 mg.	5 mg.	7 mg.	1 mg.

Unlike the other enzymes this enzyme is present in nearly the same amount in all organs. Erythrocytes contain only one-seventh as much as the other organs; serum none.

For the isolation of the enzyme lung tissue is especially suitable because of its low content of dehydrogenases [Breusch, 1937, 2].

50 g. of fresh minced lung of cats were allowed to stand for 30 min. in 200 ml. Ringer-phosphate solution, stirring from time to time. After centrifuging, the red-brown fluid was filtered through cotton-wool, precipitated with an equal volume of saturated solution of ammonium sulphate and filtered through filter paper in an ice-box. The whole active principle goes into the filtrate. The clear filtrate is saturated with ammonium sulphate, and the second newly formed precipitate filtered on filter paper in an ice-box. The precipitate is dried by pressing it between filter paper and dissolved in a Ringer-phosphate solution. A further purification can be obtained by repeated precipitation with ammonium sulphate up to 66 % saturation. By repeated precipitation with ammonium sulphate the activity decreases rapidly, perhaps owing to the elimination of a coenzyme.

In watery solution the enzyme is active at room temperature for about 24 hr., in the ice-box for some days. The pH optimum lies at the neutral point. Activity is destroyed by heating to 100°. The enzyme is not inhibited by *M*/200 arsenious acid, or by 0.1*M* NaF and *M*/1000 iodoacetic acid. *M*/1000 HCN inhibits 40 %.

The transamination is only given by free glutamic acid; glutathione, containing as an end group glutamic acid with a free amino group, does not react. As von Euler *et al.* [1939] showed, not only *l*(+)-glutamic acid, but also, to a smaller extent, *d*(-)-glutamic acid reacts.

Decarboxylation of OAA to PUA

Mayer [1914] first found that tissues contain a substance which is extractable by water, is resistant to heating and accelerates the breakdown of OAA to PUA. After having found in preliminary experiments that the whole of the active substance goes into the extract during boiling, I quantitatively investigated the ability of different organs to decarboxylate OAA to PUA.

10 g. of minced organ in 10 ml. Ringer-phosphate solution were boiled for 20 min. in a water bath, cooled, centrifuged and filtered. Of the filtrate 25 ml. were incubated at 38° with 50 mg. OAA, neutralized in 10 ml. H₂O. Every 5 min. 5 ml. were analysed for OAA. The control was done with Ringer-phosphate and OAA alone.

Under these conditions I found the power of decarboxylation to be the same for liver, muscle, kidney and brain. Extract from 1 g. of fresh tissue at pH 7.7 is able to decarboxylate 1.5–3 mg. OAA in addition to the spontaneous decarboxylation of OAA. This ability varies up to 100 % in the corresponding organs of different cats.

According to the theories of Langenbeck [1936] on enzyme models it could perhaps be expected that the carboxylase activity might be due to an amine. I therefore investigated the ability of a number of biologically important amines to effect this additional decarboxylation of OAA.

To 40 mg. neutralized OAA in 25 ml. Ringer-phosphate were added 2 mg. of the neutralized substance in 5 ml. H_2O ; incubation was at 38° and a part was analysed after every hour.

Under these conditions I found the following substances to be ineffective: adenosine, adenylic acid (from yeast and from muscle), vitamin B_1 , histamine, acetylcholine and diethylamine. A low rate of decarboxylation was shown by adenine (20 % increase) and methylamine (80 % increase).

Furthermore, I examined the influence of pH on the spontaneous decarboxylation of OAA in watery solution.

The conditions were as above; to the solution were added 5 ml. 1 % HCl, 5 ml. H_2O and 5 ml. 1 % NaOH to the respective vessels.

In acid solution decarboxylation happens about twice as quickly as in alkaline and is about 50 % more rapid than in neutral solution (Fig. 1).

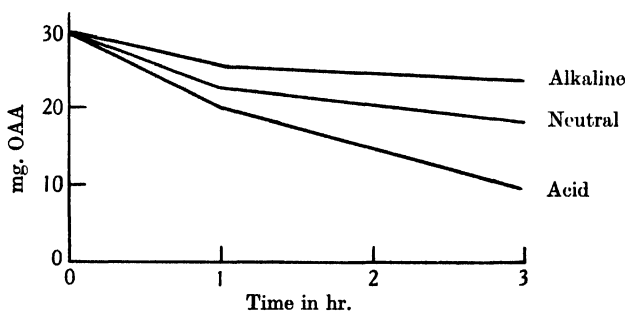


Fig. 1. Decarboxylation of oxaloacetic acid at 38° .

Wieland [1924], by measuring the CO_2 production, found no detectable decarboxylation during 2 hr. at room temperature. With this method I too found no decarboxylation: analysing the amount of OAA directly however in every case decrease of OAA was found; analysing also for PUA an equivalent increase was noted.

While neutralizing OAA in concentrated solutions, as already shown [Breusch, 1937, 1], citric acid is formed up to 10% yield by autocondensation. As Knoop has proved that citric acid is formed by shaking OAA and PUA in very weakly alkaline solution with air, it is always necessary in experiments with tissue and OAA to allow for this error by blank experiments with OAA and without tissue under the same conditions.

The neutral salts of the two isomerides of OAA, hydroxyfumaric and hydroxymaleic acid, show no difference in their behaviour in presence of tissue.

The order of loss of activity of the different enzymes

Immediately after the death of an animal there begins (under usual conditions) a slow destruction of the enzymes taking part in the metabolism of the cell.

To obtain a quantitative idea of this disturbance I investigated the stability of the enzymes under the conditions used in this paper.

5 g. of minced kidney were allowed to stand in 25 ml. Ringer-phosphate solution at 18 and 38°. After 1, 2, 3, 4 and 6 hr. the substrate to be investigated was neutralized in 5 ml. of Ringer-phosphate solution, added to the minced tissue suspension and incubated for 30 min. at 38°. The suspension was then deproteinized and analysed.

The ordinates in Figs. 2 and 3 give the amounts of substrate in mg. metabolized by 1 g. of wet tissue in 10 min.; the abscissae give the times of incubation after the death of the animal and mincing the tissue.

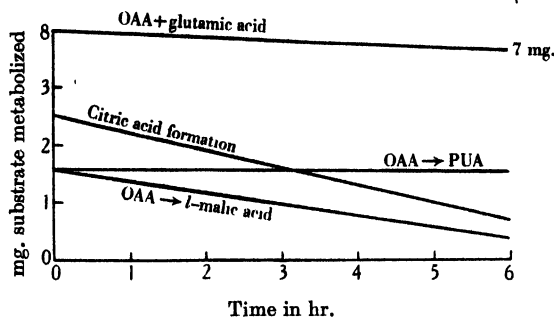


Fig. 2. At 18°.

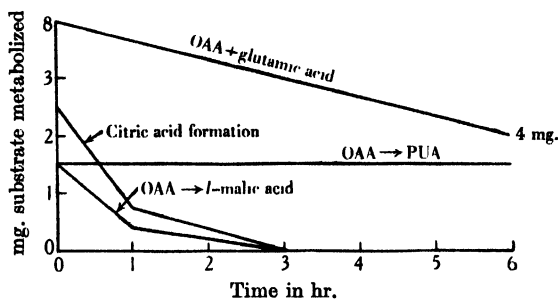


Fig. 3. At 38°.

The enzyme of OAA reduction is the most easily destroyed; that responsible for condensation of citric acid is destroyed nearly as quickly. At 38° destruction is about five times more rapid than at 18°.

The enzyme which condenses OAA with glutamic acid is more resistant. The thermostable substance which accelerates decarboxylation of OAA does not disappear to a perceptible extent under my conditions.

SUMMARY

The quantitative fate of added oxaloacetic acid has been investigated in incubation experiments, using different freshly minced tissues of cats and pigeons.

In spleen, lung, placenta and peripheral nerves added oxaloacetic acid is recovered almost quantitatively as pyruvic acid; in kidney almost quantitatively as a mixture of *l*-malic acid, pyruvic acid, lactic acid and citric acid. Deficiencies are found in muscle (about 30 %), liver (50 %), and brain (80 %).

Spleen, lung, placenta and peripheral nerves, like embryonic tissue and tumour tissue, are unable to hydrogenate oxaloacetic acid to *l*-malic acid; they merely decarboxylate the oxaloacetic acid to pyruvic acid. As they are also virtually unable to reduce pyruvic acid to lactic acid, nearly the whole oxaloacetic acid is recovered in these organs as pyruvic acid.

Muscle, liver, kidney and pancreas reduce oxaloacetic acid to *l*-malic acid, at the same time decarboxylating a part of the oxaloacetic acid to pyruvic acid and reducing this partly to lactic acid.

Brain hydrogenates oxaloacetic acid only very little. Its main action is decarboxylation and only a small part of the pyruvic acid is reduced to lactic acid.

Kidney, and to a small extent liver, brain and lung, condense oxaloacetic acid with pyruvic acid (also with glyceric acid and dihydroxyacetone) to give citric acid.

Muscle has, in confirmation of my previous work and contrary to the citric acid cycle theory of Krebs, no ability to form citric acid. It is further shown, in confirmation of perfusion experiments of Martensson, that muscle, unlike liver and kidney, has only slight ability to break down citric acid: again contrary to the cycle theory.

A new method for extraction of the enzyme which reduces oxaloacetic acid to *l*-malic acid is described. Its *pH* optimum is in the alkaline region. This enzyme is very unstable, needs coenzyme and is destroyed by heating.

The enzyme which combines pyruvic acid (to a smaller extent also glyceric acid and dihydroxyacetone) with oxaloacetic acid to citric acid in kidney, is destroyed by heating and its *pH* optimum is at the neutral point. No muscles examined (skeletal, heart and stomach of cat and pigeon), contain detectable amounts of this enzyme. I propose to call the new enzyme citrogenase.

In the competition between the reduction of oxaloacetic acid to *l*-malic acid and the formation of citric acid in kidney, the hydrogenation clearly has the advantage, as postulated by the theories of Szent-Györgyi concerning the C_4 -dicarboxylic acids as H transporters.

The citric acid formation seems to be chiefly an elimination process and happens only above a certain threshold of oxaloacetic acid; by this process the body seems to dispose of any surplus of C_4 -dicarboxylic acids, which are not excretable in the urine, whilst citric acid is readily excreted.

The enzyme for condensation of oxaloacetic acid with glutamic acid can be extracted from tissues by phosphate; it is possible to purify it by precipitation with ammonium sulphate; it is obtained in the fraction between 50 and 66 % saturation.

It is much more stable than the first two enzymes; the *pH* optimum is at the neutral point. It is destroyed by heating. Unlike the other enzymes this enzyme is found in nearly the same amount in all organs.

The thermostable substance which accelerates the decarboxylation of oxaloacetic acid to pyruvic acid is also present in all organs to about the same amount.

The order of spontaneous inactivation of these enzymes in minced kidney tissue was investigated. The enzyme whose activity disappears first is that which reduces oxaloacetic acid to *l*-malic acid; the next that condensing oxaloacetic acid with pyruvic acid to form citric acid; 5–10 times later the enzyme condensing oxaloacetic acid with glutamic acid. The thermostable substance which accelerates the decarboxylation of oxaloacetic acid to pyruvic acid remains unchanged.

The spontaneous decarboxylation in aqueous solution of the oxaloacetic acid used was examined in relation to acidity or alkalinity. In acid solution decarboxylation is twice as fast as in alkaline and 50 % more rapid than in neutral solution.

I wish to thank my collaborator Miss Paula Schwerin for the service she rendered in analytical work.

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CCXX. VITAMIN A AND CAROTENOIDS IN THE LIVER OF MAMMALS, BIRDS, REPTILES AND MAN, WITH PARTICULAR REGARD TO THE INTENSITY OF THE ULTRAVIOLET ABSORPTION AND THE CARR-PRICE REACTION OF VITAMIN A

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THE amount of vitamin A in the liver has always attracted considerable interest, partly because of the industrial production of vitamin A preparations from the liver of lower vertebrates (fish) and certain mammals (whales), partly because of the physiological interest attached to this organ. By far the greatest amount of the body's vitamin A reserves are stored in it, and in this organ carotene is converted into vitamin A.

Using the SbCl_3 method of assay, large series of estimations of the amount of vitamin A in the human liver [Wolff, 1932; 1936; 1938; Moore, 1937] and the liver of common domestic and laboratory animals [e.g. Sen & Sharma, 1936; Holmes *et al.* 1936] have already been carried out. In the case of wild animals, however, only meagre data are available. Nevertheless, such investigations may be expected to furnish items of considerable interest, as the relation between the various kinds of food and environment on the one hand and the vitamin A content of the liver on the other, may yield important information on the physiology of the vitamin. The possible presence of the so-called vitamin A_2 and other vitamin A factors in certain species might cast some light on the role these substances play in the metabolism of vitamin A and the carotenoids in these animals.

In addition to these strictly biological problems, the quantitative estimation of vitamin A in liver tissue still lacks perfection.

Most investigators have used the SbCl_3 method when working on the liver of warm-blooded animals. This method is not commonly regarded as being quite accurate for determining the absolute quantity of vitamin A, as the conversion factor for expressing the strength of the reaction in terms of international units (i.u.) of vitamin A is liable to wide variations. While thus the factor $E_{1\text{cm}}^{1\%} = 1$ corresponding to 1600 i.u. per g. has been internationally accepted for the absorption at 328 μ , there is no similar international factor for the Carr-Price reaction [Hume & Chick, 1935; Wolff, 1938]. It is, therefore, necessary to determine the conversion factor of the Carr-Price reaction for each source of vitamin A if the results are to be expressed in i.u. The factor is to be determined by comparing the Carr-Price values and the results of biological or spectrographic estimations of vitamin A.

We have not succeeded in finding in the literature a conversion factor for the Carr-Price reaction of mammalian and bird livers; spectrographic determination of these sources of vitamin A appears to have been carried out in a few

instances only. Thus Capper [1930] and Capper *et al.* [1931] have made spectrographic estimations of the unsaponifiable matter of rat and fowl livers respectively. The curves obtained showed a maximum at 328 $m\mu$ and, on the whole, they resembled the curves obtained for fish liver oils. These authors, however, did not employ the extinctions as a quantitative measure of vitamin A; the spectrographic determination was used only as proof of its presence.

As far as we know, quantitative spectrographic analysis of the vitamin A content of the liver of warm-blooded wild animals and man has not been attempted. Some of the latest and most extensive work has been reported by Gillam [1938] and Karrer *et al.* [1932], who examined, respectively, 21 and 34 different vertebrates; however, these authors give their results only in terms of the Carr-Price reaction.

In order to find a conversion factor for the Carr-Price reaction of livers we have deemed it necessary to determine the Carr-Price reaction as well as the ultraviolet absorption of the liver extracts in a large number of mammals and birds representing as many zoological groups as possible; stress has accordingly been placed on the comparative study of the two methods. Also, the carotenoids have been examined chromatographically, as up to the present a few species only have been studied satisfactorily by this method [Zechmeister & Tuzson, 1935]. In the biological discussion of the results of our analyses, it will be seen that it is not possible to draw general conclusions regarding the normal vitamin A or carotenoid (carotene) content of livers from individuals of any one species, though, apparently, individual variations are smaller than variations from one species to another.

MATERIAL

Most of the animal livers examined were obtained from the Zoological Museum of the University; the livers of domestic animals were purchased at a butcher's; livers of game were obtained through acquaintances and those of cats and dogs from the Royal Veterinary School; human livers came from the Medico-Legal Institute of the University and the Pathological Department of the Bispebjerg Hospital.

The liver specimens were saponified immediately on receipt, or were stored in the refrigerator not more than 12 hr. In several cases, 2 days elapsed from the death of the animal to saponification, as some animals were killed far from Copenhagen, e.g. in Jutland. With very few exceptions, the interval was never more than 72 hr.

EXPERIMENTAL METHODS

(1) *Samples*

Small livers (under 100 g.) were examined *in toto*; in the case of larger ones pieces from different parts of the organ were taken to ensure an average sample. This procedure was adopted as rather marked differences in the concentration of vitamin A in different parts of the same liver were observed; Moore [1937] states that the concentration in all the hepatic lobes is practically the same, although he does not give details. Sen & Sharma [1936] found higher values generally in the central parts of the liver than in the peripheral; the difference, however, did not amount to more than 10%. Lindqvist [1938], in a series of 22 duplicate analyses on different samples of the same lobe, found a standard deviation of 3.78 on a single analysis, while differences between the right and

left human lobes amounted to 75%. These workers all used the technique of Davies [1933] for extraction and measurement of the Carr-Price reaction (the two first used the Lovibond tintometer, the last-named the Pulfrich photometer).

(2) *Saponification and extraction*

Saponification and extraction were performed by a modification of the technique of Davies [1933]. Small pieces of liver (each under 5 g.) were prepared and weighed together. 4 ml. 5% alcoholic KOH were used per g. liver and the mass was heated $\frac{1}{2}$ hr. on a boiling water bath under a reflux condenser. An addition of about $\frac{1}{3}$ volume light petroleum¹ to the mixture gave prompt boiling and accelerated the solution of the tissue. After saponification, an equal volume of distilled water was added. The extraction was performed 3–5 times until the extract was colourless, using about $\frac{1}{2}$ volume of peroxide-free ether. The combined extracts were washed 3–5 times with a liberal amount of distilled water and dried by suction through a 3–5 cm. layer of anhydrous Na_2SO_4 . The extract was then evaporated to dryness in an atmosphere of N_2 ; the remainder was dissolved in light petroleum and transferred to a graduated flask (5–50 ml., according to the amount of liver). A certain amount (usually half) of the solution was evaporated, the residue dissolved in absolute alcohol for spectrographic determination and the remainder used for measuring the Carr-Price reaction, the yellow value (total carotenoid content) and chromatography.

(3) *Spectrographic determination*

Spectrographic measurements were made with a Hilger E_3 quartz spectrograph with Echelon cell outfit following the method of Twyman [1933]. This equipment allows for rapid exposures and wide extinction range.

In making the spectrograms, three values of density were chosen so that the 30 pairs of spectra constituted a series of variations in which the logarithms of the extinctions calculated for 1 cm. layer of thickness varied for each step between the limits +0.07 and +1.08.

Absolute ethanol was used as a solvent throughout, the strongest solution possible being used for the first exposures, and when dilutions were necessary the dilutions 1 : 10 and 1 : 100 were used. By adopting this procedure, the parts of the curve represented by two consecutive plates lie in continuance of each other and the logarithms of the extinction values of the dilution 1 : 10 are comparable with those of the stock solution simply by addition of the figure 1, and so on. The match points were marked and the readings taken by two observers independently of each other. Thus a very ample and very exact representation of all parts of the extinction curve was obtained in the range of about 200–500 $\text{m}\mu$ if the original concentration was strong enough.

The graphs were plotted with the wave-length along the abscissa (1 cm. \sim 20 $\text{m}\mu$) and the logarithm of the extinctions for 1 cm. thickness of liquid as ordinate (1 cm. $\sim \log \epsilon = 0.20$). The extinction curves should become uniform by this arrangement, i.e. only the height and not the shape of the curve depends on the concentration.

The extinction at 328 $\text{m}\mu$ and 450 $\text{m}\mu$ at the given concentration was read off on the curves, and the values $E_{1\text{cm}}^{328}$, 328 $\text{m}\mu$ and $\frac{e_{328\text{m}\mu}}{e_{450\text{m}\mu}}$ may then be calculated. Unfortunately, lack of space prevents us from giving illustrations of any of the graphs.

¹ The light petroleum had a B.P. of 65–70°.

(4) *Measurement of the Carr-Price reactions*

These measurements were made on a Zeiss Pulfrich photometer with a $\frac{1}{2}$ cm. cell. The stock solution was progressively diluted until the extinction lay between 0.15 and 0.50. 0.2 ml. of the solution was used for the reaction; 2 ml. Carr-Price reagent were added prior to which 1 drop (never more than 2 drops) of acetic anhydride was placed in the cell (details of the technique are given by Willstaedt & Jensen [1937]). The readings were taken as soon as possible after addition of the reagent, i.e. after about 5 sec.; 2-5 samples were measured, the cells interchanged, and corresponding readings taken on the other side. The mean value of the measurements has been given as the result.

The extinction coefficient of the blue reaction was calculated as follows¹:

$B_{1\text{ cm}}^{1\%} = \frac{E_n}{s \times c} \times E_n$ denotes the mean value of n measurements (half from each side of the photometer) of a 0.2 ml. solution + 2 ml. reagent; s denotes the layer of thickness expressed in cm., i.e. $\frac{1}{2}$; c is the concentration expressed in g. per 100 ml. of the solution in the cell (the original solution + the reagent), and therefore 1/11 of the concentration of the original solution.

The value of the blue colour readings with the various filters is designated by S 59, S 61 and S 66.6 (see Tables).

(5) *Analysis of the carotenoids*

As the amount of carotenoid in the liver is generally very small, the quantitative estimation was limited to the determination of the so-called "yellow value" (total carotenoid) and to the quantitative estimation by microchromatography.

The yellow value was measured on the Pulfrich photometer with the filter S 43, in several cases also with the filters S 45 and S 47. The values are given in the Tables as $C_{1\text{ cm}}^{1\%}$, the number of the filter being stated. In some cases in which the material was insufficient for exact measurement only the upper limit has been given.

Chromatographic measurements were carried out by the technique of Willstaedt & With [1938], i.e. Al_2O_3 purissimum "Merck" and columns 2-5 mm. wide and 5-7 cm. in height; development was performed with benzene-petroleum (B.P. 65-70°) 1 : 1 or 2 : 1 or pure benzene.

From the appearance of the microchromatograms it could generally be judged with certainty if any considerable amount of carotene were present; amounts of carotene below 10-15 %, as well as the nature of the other bands, could merely be conjectured. In many cases, clear yellow bands were found at the top of the column; these were very likely due to phytoanthin, which was rarely present in any considerable amount. In most cases brownish or brownish-yellow bands, presumably due to oxidation products of the carotenoids, were found lower down. The carotene appeared as a rose-coloured zone partly or entirely passing into the filtrate; this zone in concentrations above 30 % was well marked. Occasionally a yellow zone (? α -carotene) might be distinguished below. Other zones, from red to rose-coloured, might appear below the oxidation products, sometimes separated from these by colourless bands; they were never diffuse like the carotene zone. The exact nature of these zones could not be determined by micro-analysis.

¹ This method of calculation is allowable as the Carr-Price reaction read in a spectrograph or a spectrophotometer follows Beer's law [Notevarg & Weedon, 1936; Dann & Evelyn, 1938].

By painting the column with Carr-Price reagent [Zechmeister *et al.* 1936] the presence of vitamin A might be detected. If carotene were present in measurable amount, the blue colour stopped before the carotene zone, while the other zones, especially those of oxidation products, as a rule were coloured intensely blue.

In some chromatograms of livers rich in vitamin A, no distinct colour zones appeared; instead there was a cloudy discoloration of the whole column with an intense dark blue Carr-Price reaction. However, this occurred only in livers rich in vitamin A that exhibited a low yellow value.

Comparison of the yellow values found with S 43, S 45 and S 47 often gave a valuable hint of the nature of the carotenoids present, as the values are of the same order, more or less, for carotene, xanthophyll, zeaxanthin and kryptoxanthin, while for lycopin the extinction value with S 47 is considerably higher than with S 43, and the oxidation products often show great differences in the extinction values with the filters mentioned.

(6) *Investigation of the loss due to time*

Davies [1933] was not able to demonstrate any loss after keeping his samples for 3 days at room temperature. We also have investigated the problem, as will appear below. After mincing and weighing portions of 10 g. (± 0.1 g.), 2 portions were analysed immediately, 2 after standing 2 days in the dark in a closed vessel at room temperature, 2 portions after standing 4 and 2 after standing 8 days. The extinctions of the extracts of 10 g. liver made up to 5 ml. are given below. Microchromatography revealed that practically all the carotene was β -carotene. It will be seen that under these conditions the loss of vitamin A and of carotene does not exceed 10% in 8 days. As these experimental conditions closely resemble the condition of the vitamin A in the liver of the dead animal, it may be concluded that no significant loss has occurred due to keeping.

	Before	2 days	4 days	8 days
Carotene (S 43; $\frac{1}{2}$ cm.)	0.310	0.319	0.297	0.295
	0.323	0.314	0.292	0.288
Vitamin A (S 61; $\frac{1}{2}$ cm.; 0.2 ml. sol. + 2.0 ml. reag.)	0.224	0.228	0.221	0.186
	0.208	0.207	0.204	0.206

Explanation of the Tables

In the Tables the results have been schematically arranged for mammals (Table I), birds (Table II), reptiles (Table III) and the human subject (Table IV).

Column 1 gives the systematic zoological order, column 2 the name of the species. Columns 3, 4 and 5 give the extinctions " $B_{1\text{cm}}^{1\%}$ " ($E_{1\text{cm}}^{1\%}$ for the Carr-Price reaction) with the three filters used. Column 6 gives the extinction " $E_{1\text{cm}}^{1\%}$ " at 328 $m\mu$ of vitamin A. To give some idea of the form of the extinction curve, the absorption maxima are set up in column 14. Figures without any parentheses denote a clear maximum represented by a peak on the curve, single parentheses denote an inflexion on the curve and double parentheses signify that the curve at the given wave-length has a slanting direction without any sign of a maximum or inflexion. Similarly, we have tried to give briefly a rough idea of possible variations in the Carr-Price reaction extinction curve in columns 8 and 9 ($B_{1\text{cm}}^{1\%}$ S 61 = 1); in column 8 is given the extinction with filter S 59 divided by the extinction with S 61, and in column 9 the extinction value with the filter S 66.6 is divided by the extinction with S 61. Column 7 gives the relations $\frac{e_{328\text{m}\mu}}{e_{450\text{m}\mu}}$

between the extinctions at 328 $m\mu$ (the vitamin A absorption maximum) and 450 $m\mu$ (the absorption maximum of the carotenoids), as read off on the spectrographic curves. The relation has been termed larger than ($>$) a certain value if the extinction at 450 $m\mu$ has been less than the smallest extinction which can be read at the greatest concentration in which an exposure has been possible. Column 10 gives the relation " $B_{1\text{ cm}}^{1\%} \text{ S 61} : E_{1\text{ cm}}^{1\%} \text{ 328 } m\mu$ " between the extinction coefficient of the Carr-Price reaction at its maximum (S 61) and the extinction coefficient of vitamin A at 328 $m\mu$; this ratio is of great importance in determining the utility of the Carr-Price reaction as a quantitative method of assay of vitamin A. In the remaining columns, 11 and 12, the carotenoid extinction is given per g. in 100 ml. in 1 cm. layer " $C_{1\text{ cm}}^{1\%}$ " with the filters S 43 and S 47. Column 13 shows the results of the microchromatographic estimation of carotene.

The results obtained with human livers are in the main presented in the same way; columns 1-3 furnish data such as protocol number, sex, age and autopsy findings. The other columns are identical; the column dealing with spectral maxima has been omitted, as all the livers showed a clear maximum at 328 $m\mu$.

Owing to prevailing circumstances, all the determinations were not carried out on some livers.

The results have not been converted into micrograms carotene or to I.U. of vitamin A, the extinction coefficient $E_{1\text{ cm}}^{1\%}$ having been preferred as a basis for the quantitative estimations.

With the help of the Tables it is easy to convert the absorption at 328 $m\mu$ into I.U. per g. liver, the value $E_{1\text{ cm}}^{1\%}$ having simply to be multiplied by 1600; however, this obtains only when the curve has a clear maximum at 328 $m\mu$ or thereabout. The conversion of the carotenoid value into micrograms may be performed by using the current value of the extinction coefficient $E_{1\text{ cm}}^{1\%}$ of carotene with the given filters; this conversion factor ranges from 1800 to 2500, according to different investigators. The following values for β -carotene were reported by Willstaedt & Jensen [1939, 2]: $C_{1\text{ cm}}^{1\%} \text{ S 43} = 1963$, $C_{1\text{ cm}}^{1\%} \text{ S 45} = 2352$, $C_{1\text{ cm}}^{1\%} \text{ S 47} = 2242$.

Physico-chemical discussion

From the commencement of this work, the main interest has been centred on the relation between the extinction coefficients of the Carr-Price reaction and of the ultraviolet absorption, because, as mentioned in the introduction, the value of the Carr-Price reaction as a quantitative method of assay of vitamin A depends on it. The computed mean values are practically the same in the series (column 10) of values found in the mammals, birds and human subjects examined (2.60-2.75), and the standard deviation is fairly small (coefficient of variation about 15%). Thus this interesting relation is found not only in different individuals of the same species, but in all warm-blooded animals, in spite of the great variation in the quality of their food.

The animals that did not show a definite maximum on the absorption curve at 328 $m\mu$ have been omitted from the results, as the absorption at this wavelength in these cases was due not only to vitamin A,¹ but also to other causes such as the presence of carotenoid.

¹ The considerable discrepancy in the relation $B_{1\text{ cm}}^{1\%} \text{ S 61} : E_{1\text{ cm}}^{1\%} \text{ 328 } m\mu$ found in the white-nosed grivet must be regarded as being due to the technical error of omitting to record the dilution 1:10 of the solution employed in the Carr-Price reaction. We have, therefore, placed the value found in parentheses, and in our calculations reckoned with 10 times the amount.

Table I. *Mammals*

1	2	3-5		6	7	8-9		10	11-12		13	14
Order	Species	$E_{1\text{cm}}^{1\text{cm}}$		$E_{1\text{cm}}^{1\text{cm}}$ 328 mμ	$\epsilon_{550\text{ m}\mu}$ $\epsilon_{450\text{ m}\mu}$	$E_{1\text{cm}}^{1\text{cm}}$ S 59	$E_{1\text{cm}}^{1\text{cm}}$ S 61=1	$E_{1\text{cm}}^{1\text{cm}}$ S 61 $E_{1\text{cm}}^{1\text{cm}}$ 328 mμ	S 43	$C_{1\text{cm}}^{1\text{cm}}$ S 47	Carotene (micro- chromato- graphic estimation)	Spectra maxima (mμ)
Cetacea	Grampus (<i>Grampus griseus</i>)	I. 0.0418	No reaction	0.04174	0.0191	—	—	—	—	—	0	—
	Mole (<i>Talpa europaea</i>)	II. 0.0065	0.125	—	—	—	0.403	—	0.0012 <0.002	0.0011	0	—
Chiroptera	Bat (<i>Vesperugo</i> sp.)	—	1.16	—	—	—	—	3.02	0.014	0.009	0	325
Rodentia	House-mouse (<i>Mus musculus</i>)	—	No reaction	<0.023	—	—	—	—	<0.005	—	0	—
	Squirrel (<i>Sciurus vulgaris</i>)	0.161	0.197	0.076	—	0.114	0.817	0.386	<0.002	—	0	330 (450)
	Hare* (<i>Lepus timidus</i>)	I. 0.495	0.703	0.087	—	0.236	0.704	0.124	0.0022	0.0012	0	325 (450)
	Guinea-pig (<i>Cavia porcellus</i>)	II. 0.238	0.329	0.046	—	—	0.723	0.140	0.0016	0.0013	—	—
		I. 0.0036	0.0044	0.0009	—	0.0038	0.817	0.208	0.0012	0.0039	0	(328) 450
		II. —	—	—	—	0.0024	3.72	—	—	—	—	(280) (328) (390)
	Capibara (<i>Hydrochoerus capivara</i>)	0.176	0.247	0.035	—	0.097	0.713	0.142	<0.0004	—	0	325
Carnivora	Cat (<i>Felis catus</i>)	I. 0.496	0.727	0.106	—	0.324	0.682	0.146	0.0048	0.0040	About 40%	328 (450)
		II. 0.440	0.686	0.120	—	0.237	0.641	0.175	0.0036	0.0036	—	326 (450)
	Dog (<i>Canis familiaris</i>)	I. 0.334	0.449	0.106	—	0.153	0.744	0.236	0.0018	0.0012	0	324 (450)
		II. 0.0775	0.109	0.033	—	0.038	0.711	0.303	0.0012	0.0010	Trace	324
		III. —	0.060	—	—	—	—	—	0.0032	0.0027	—	—
		IV. —	0.172	—	—	—	—	—	0.0022	0.0016	—	—
	Badger* (<i>Meles meles</i>)	0.0606	0.0756	0.0133	—	0.0308	0.771	0.169	0.008	—	About 60%	283 322 450
	Fox (<i>Canis vulpes</i>)	I. 1.37	1.78	0.352	—	0.641	0.770	0.198	0.008	0.0153	About 33%	325 (450)
		II. 2.81	3.90	0.569	—	1.54	0.720	0.146	0.0120	0.0109	About 40%	325 (450)
		III. —	0.450	—	—	—	—	—	0.0112	0.0102	About 60%	—
	Ermine (<i>Mustela erminea</i>)	I. 0.0735	0.102	0.0092	—	0.0532	0.721	0.090	<0.0005	—	0	300 (328)
		II. 0.121	0.139	0.046	—	0.104	0.871	0.331	<0.002	—	0	300 (328)
	Fitchet (<i>Mustela putorius</i>)	I. 1.05	1.50	0.328	—	0.562	0.700	0.219	0.013	0.013	About 30%	326 (450)
		II. 1.82	2.46	0.354	—	0.800	0.740	0.144	0.009	0.008	About 60%	325 (450)
	Otter (<i>Lutra lutra</i>)	1.83	2.78	0.824	—	1.096	0.658	0.297	0.007	0.003	0	325 (450)
Ungulata	Swine (<i>Sus scrofa domestica</i>)	0.122	0.172	0.039	—	0.0621	0.709	0.227	<0.0002	—	0	326
	Horse (<i>Equus caballus</i>)	0.644	1.08	0.360	—	0.308	0.692	0.333	0.0055	0.0054	About 80%	325 450 (475)
	Cow* (<i>Bos taurus</i>)	I. 0.570	0.817	0.167	—	0.296	0.698	0.204	0.0055	0.0054	About 80%	325 450 (475)
		II. 0.0756	0.0969	0.0168	—	0.0401	0.782	0.173	—	—	—	325 450
	Sheep (<i>Ovis aries</i>)	1.77	2.47	0.540	—	0.957	0.717	0.219	—	—	—	325 450
	Roedeer (<i>Capreolus capreolus</i>)	1.40	2.29	0.265	—	0.900	0.611	0.116	0.0074	0.0065	About 30%	324 (450)
Primates	Whitenoosed grivet (<i>Cercopithecus mitis</i>)	0.0234	0.0275	0.0096	—	0.108	0.851	0.349	0.003	—	0	322
		—	—	—	—	—	—	—	—	—	—	—

* Not included in the calculation of M and σ because the liver was received after the termination of the original series and the calculation of its mean values.
† I, adult cow; II, calf.

Table II. *Birds*

1 Order	2 Species	3-5 $B_{1cm}^{1/2}$		6 $E_{1cm}^{1/2}$ 328 mμ	7 $\epsilon_{298m\mu}$ $\epsilon_{430m\mu}$	8-9 $B_{1cm}^{1/2}$ S 61 = 1		10 $B_{1cm}^{1/2}$ S 61 $E_{1cm}^{1/2}$ 328 mμ		11-12 $C_{1cm}^{1/2}$		13 Carotene (micro- chromato- graphic estimation)	14 Spectral maxima (mμ)
		S 59	S 61			S 59	S 66.6			S 43	S 47		
Colymbiformes	Polar loon (<i>Colymbus arcticus</i>)	—	2.75	—	—	—	—	—	—	0.009	0.005	0	—
	Cormorant (<i>Phalacrocorax carbo</i>)	0.174	0.251	0.092	0.107	0.693	0.367	2.35	0.0197	0.0194	0	0	326 (450) (470)
Steganopodes	Gannet (<i>Sula bassana</i>)	4.56	6.65	1.34	2.15	0.686	0.202	3.10	0.0058	0.0044	0	0	326 (450)
	Velvet-duck (<i>Oidemia fusca</i>)	0.196	0.249	0.049	0.0928	0.787	0.197	2.69	0.014	0.013	0	0	(290) 328 450
Lamellirostres	Garganey (<i>Anas querquedula</i>)	0.202	0.213	0.059	0.126	0.948	0.277	(1.69)	0.127	0.135	About 50 %	Trace	280 (328) 455
	Wild duck (<i>Anas boschas</i>)	0.171	0.261	0.054	0.0886	0.655	0.207	2.94	0.013	0.013	Trace	Trace	328 455
	Teal (<i>Anas crecca</i>)	0.289	0.370	0.065	0.196	0.761	0.176	1.89	0.011	0.012	Trace	Trace	285 324 (450)
	Merlin (<i>Falco aesalon</i>)	0.137	0.177	0.025	0.0695	0.774	0.141	1.98	<0.0006	—	0	0	320
Raptatores	Kestrel (<i>Falco tinnunculus</i>)	2.71	3.75	0.674	1.31	0.723	0.180	2.66	0.018	0.014	0	0	325 (450)
	Shaggy-footed buzzard (<i>Buteo lapogus</i>)	6.16	9.12	1.68	3.21	0.675	0.184	2.85	0.009	0.006	0	0	328 ((450))
	Buzzard (<i>Buteo buteo</i>)	I. 2.13 II. —	3.06 2.51	0.415 —	0.953 —	0.686	0.136	3.21	0.003 0.023	— 0.017	0 15-20 %	0 15-20 %	326 —
	Curassow (<i>Crax fasciolatus</i>)	0.0228	0.0250	0.0099	0.0226	0.912	0.396	(1.11)	0.031	0.027	About 10 %	About 10 %	450
Galliformes	Eared pheasant (<i>Crossoptilon auritum</i>)	—	0.219	—	—	—	—	—	0.0043	0.0039	Trace	Trace	—
	Black grouse (<i>Tetrao tetrix</i>)	3.52	4.39	1.03	1.61	0.802	0.235	2.73	0.019	0.019	0	0	324 (450)
	Hen (<i>Gallus domesticus</i>)	I. 0.121 *II. — III. — IV. —	0.169 0.550 0.123 6.85	0.037 — — —	0.0662 0.230 0.063 3.55	0.716 — — —	0.219 — — —	2.56 1.95 1.92	— 0.016 0.002	— — —	— — —	— — —	326 445 — —

Balliiformes	Water rail (<i>Rallus aquaticus</i>)	—	0-458	—	—	—	—	—	0-039	0-026	0	—
	Common coot (<i>Fulica atra</i>)	0-0509	0-0676	0-0161	0-0364	2-24	0-753	0-238	1-86	0-016	Trace	324 450
	Water hen (<i>Gallinula chloropus</i>)	—	0-322	—	—	—	—	—	—	0-036	About 50 %	—
	Stone plover (<i>Oedicnemus creptans</i>)	—	0-426	—	—	—	—	—	—	0-005	0	—
Limicolae	Snipe (<i>Gallinago scolopacea</i>)	0-692	0-904	0-184	0-306	>12-9	0-765	0-204	2-96	0-0143	Trace	325
	Dottrel plover (<i>Eudromias morinellus</i>)	0-106	0-141	0-023	0-0914	>5-13	0-766	0-163	(1-54)	0-011	0	(290) (326)
	Golden plover (<i>Charadrius apricarius</i>)	—	0-0158	—	0-0304	>3-02	—	—	(0-52)	<0-003	0	(290) (326)
	Silver gull (<i>Larus argentatus</i>)	1-73	2-35	0-631	0-756	331	0-736	0-269	3-11	0-005	0	325 (450)
Laridae	Arctic gull (<i>Stercorarius parasiticus</i>)	1-03	1-48	0-274	0-483	79-4	0-696	0-185	3-06	0-006	0	326 (450)
Striges	Tawny owl (<i>Strix aluco</i>)	0-916	1-24	0-192	0-417	>33-9	0-739	0-155	2-97	0-0077	0	328
	Screech owl (<i>Athene noctua</i>)	0-494	0-635	0-181	0-222	>16-2	0-778	0-285	2-85	0-0036	0	320
	Short-eared owl (<i>Otus flammea</i>)	—	0-475	—	—	—	—	—	—	0-0111	0	—
	Toucan (<i>Rhamphastos</i> sp.)	—	—	—	—	—	—	—	—	0-011	0	—
Scansores	Cuckoo (<i>Cuculus canorus</i>)	0-335	0-479	0-133	0-171	5-75	0-669	0-278	2-80	0-0067	0	320
	Amazon parrot (<i>Amazona aestiva</i>)	—	0-013	—	—	—	—	—	—	0-0022	0	—
	Starling (<i>Sturnus vulgaris</i>)	—	0-709	—	—	—	—	—	—	0-028	0	—
Passeres	Thrush (<i>Turdus musicus</i>)	0-478	0-590	0-110	0-209	>4-68	0-810	0-186	2-83	<0-005	0	325
	Red-start (<i>Ruticilla phoeniceus</i>)	—	0-514	—	—	—	—	—	—	0-018	0	—
	Willow-wren (<i>Phylloscopus trochitis</i>)	—	0-323	—	—	—	—	—	—	0-060	Trace	—
	Kinglet (<i>Regulus cristatus</i>)	—	0-884	—	—	—	—	—	—	0-141	Trace	—
	Lark (<i>Aldaia arvensis</i>)	I. — II. —	1-159 0-639	— —	— —	— —	— —	— —	— —	0-032 0-027	0 0	— —
							M: 0-753 σ: 0-085	0-223 0-062	2-63 0-436			

* II, III and IV are medians of groups each of 5 hens.

Table III. *Reptiles*

1	2	3-5	6	7	8-9	10	11-12	13					
Order	Species	$B_{1\text{cm}}^{1/2}$	$E_{1\text{cm}}^{1/2}$	$e_{338\text{ m}\mu}$ $e_{430\text{ m}\mu}$	$B_{1\text{cm}}^{1/2}$ S 61=1	$B_{1\text{cm}}^{1/2}$ S 61 $E_{1\text{cm}}^{1/2}$ 328 m μ	$C_{1\text{cm}}^{1/2}$	Carotene (micro- chromato- graphic estimation)					
Ophidae	Viper (<i>Vipera berus</i>)	S 59	S 61	S 66-6	S 59	S 66-6	S 43	S 47					
		I.	—	0-0331	—	—	—	—	0				
		II.	—	0-016	—	—	—	—	0				
		III.	—	0-016	—	—	—	—	0				
Varanus	Monitor (<i>Varanus comodensis</i>)	1-60	2-15	0-324	0-822	159	0-744	0-152	2-62	0-023	0-017	10-30 %	328

Table IV. *Human livers*

1	2	3	4-6	7	8	9-10	11	12-14	15
Sex and age	Diagnosis (post-mortem examination)	$B_{1\text{cm}}^{1/2}$	$E_{1\text{cm}}^{1/2}$	$e_{338\text{m}\mu}$ $e_{430\text{m}\mu}$	$B_{1\text{cm}}^{1/2}$ S 61=1	$B_{1\text{cm}}^{1/2}$ S 61	$E_{1\text{cm}}^{1/2}$ S 61	$C_{1\text{cm}}^{1/2}$	Carotene (micro- chromo- graphic estimation)
I.	Pneumonia	S 59 S 61	S 66-6 S 66-6	0-0546 0-0546	S 59 S 66-6	$E_{1\text{cm}}^{1/2}$ S 61 $E_{1\text{cm}}^{1/2}$ S 61	2-40	S 43 S 47	—
II.	Aneurism	0-107 0-278	0-131 0-370	6-17 11-5	0-817 0-751	0-305 0-178	2-32	—	—
III.	Pneumonia. Mb. cordis	0-0716	0-0980	3-89	0-731	0-195	2-73	0-0151	About 50%
IV.	Cirrhosis hepatis. Osteo- malacia. Steatorrhoea	0-0179	0-0226	>4-47	0-792	0-208	(1-79)	0-0113 0-0006	About 50% Trace
V.	Embolism	0-762	1-06	0-302	0-719	0-215	3-52	0-009	About 30%
1.	Suicide	0-336	0-482	0-185	0-697	0-187	2-60	0-021	About 70%
2.	Mb. cordis	0-269	0-368	0-138	0-693	0-188	2-82	0-0045	About 40%
3.	Mb. cordis	0-218	0-316	0-120	0-691	0-145	2-62	0-0149	About 50%
					M: 0-736 σ: 0-050	0-203 0-022	2-75 0-406		

The presence of greater amounts of carotenoids appears in the ratio $e_{328\text{ m}\mu} : e_{450\text{ m}\mu}$ (column 7) which is particularly small in these cases, i.e. the garganey, the currawong and the guinea-pigs.

It seems that the absorption curves showing a maximum neither at 328 m μ nor at 450 m μ are due to peculiarities of the species; they have been found in the two ermines, the golden plover, the dotterel plover and partly also in the squirrel, in which the curve shows a maximum at 320–330 m μ , but is conspicuously flat. One of the human livers (no. IV) is also an exception; this must be ascribed to a pathological condition, as this person suffered from a severe hepatic disorder with fatty diarrhoea; this furnishes a plausible explanation of the peculiarities of the curve and the low concentration of vitamin A and carotene (cf. Table IV).

The relation between the ultraviolet absorption at 328 m μ of vitamin A and the absorption of carotenoids in the maximum wave-length (near 450 m μ) (see column 7 in the Tables) varies considerably; this must be attributed to the great variation in the amount of carotenoid in the food of the different species, as the amount of carotenoid in the liver depends in the first instance on the carotenoid content of the food; this is converted into vitamin A in the liver, metabolized or stored in small quantities. The carotenoids found in the liver are, therefore, as stated above, for the most part non-characteristic metabolic products, and the amounts found are not necessarily considerable even though the animal receives a rich supply of carotenoids with the food. The concentration in the liver depends on two factors, i.e. the amount ingested and the metabolic processes in the liver. Considerable amounts have been found only in a few cases, namely in the garganey, the currawong and the guinea-pig; the extinctions due to carotenoids in these cases were of the same magnitude as the ultraviolet absorption of vitamin A. In the other livers examined, the carotenoid concentration has been so small in comparison with the concentration of vitamin A that the ultraviolet absorption due to the former has caused no error in the spectrographic measurement of the latter.

As stated above, the curves obtained for vitamin A absorption in the ultraviolet range are, with the exceptions thus mentioned, practically identical in all cases.

The absorptions of the Carr-Price reactions also show comparatively slight variation, as the extinction with S 59 divided by the extinction S 61 shows a mean value of 0.73–0.76 with a coefficient of variation of 5–10%. However, there is a considerably larger variation in the relation between the extinctions with S 66.6 and S 61, namely, a mean variation of 0.20–0.22, and a coefficient of variation between 20 and 40% (in the human material only 10%)—a natural consequence of the fact that the filter S 66.6 corresponds to that part of the absorption curve which is far from the maximum, and in which, therefore, measuring takes place in a strongly sloping part of the curve.

The form of the spectral absorption curve and the relation of the Carr-Price reaction extinctions with S 61 and S 66.6 furnish information as to the presence of vitamin A₂ and possibly other vitamin A factors, e.g. the factors found by Pritchard *et al.* [1937] in mammalian livers and by Willstaedt & Jensen [1939, 1] in whale liver oil. As the vitamin A₂ absorption maxima lie on the 340–350 and 280–290 m μ ranges, and the factors of Pritchard *et al.* [1937] as well as the factors of Willstaedt & Jensen [1939, 1] at 290 m μ , the presence of these factors in considerable amount would result in maxima or inflexions at these wave-lengths, or at least give a flattened curve. These phenomena have not been observed by us.

As classical vitamin A has its maximum in the Carr-Price reaction at 610–620 $m\mu$, while vitamin A₂ shows strong absorption bands at 690–700 $m\mu$, and the factor of Pritchard *et al.* [1937] shows two bands at 490 and 590 $m\mu$ and that of Willstaedt & Jensen [1939, 1] a band near 570 $m\mu$, the presence of the first substance should be revealed by an increase of the ratio $B_{1\text{ cm}}^{1\frac{1}{2}\%}$ S 66:6 : $B_{1\text{ cm}}^{1\frac{1}{2}\%}$ S 61 and the latter by an increase in the ratio $B_{1\text{ cm}}^{1\frac{1}{2}\%}$ S 59: $B_{1\text{ cm}}^{1\frac{1}{2}\%}$ S 61. This we have not observed. True, one ermine showed a maximum at 300 $m\mu$ and the other had a inflexion here, while the maximum at 328 $m\mu$ was absent; presumably this was due neither to the factor of Pritchard *et al.* nor to the factor of Willstaedt & Jensen, as the Carr-Price reactions were not characteristic of these substances. It has not been possible to determine the nature of these variations as the samples at our disposal were insufficient for fractionating and purifying possible new vitamin A factors. Thus other factors have not been demonstrated even in the otter, in which Gillam [1938] has been able to find vitamin A₂. It may be mentioned that the otter examined by us was a wild specimen shot by a stream in Mid-Jutland; it had, therefore, presumably fed mainly on fresh-water fish.

BIOLOGICAL DISCUSSION

The great differences in the vitamin A contents of the livers of different species are shown in the Tables. These differences are often considerable in species closely related both zoologically and with regard to the nature of their food. The variations between different individuals of the same species are unfortunately insufficiently illustrated by the present material; as far as can be seen, however, the variation from individual to individual in the same species, even though considerable at times, is nevertheless of a different magnitude from the variation from one species to another. Some examples will be given. The gannet and the cormorant are closely related zoologically and both feed on marine fish (the gannet, however, only feeds on surface fish as it is a plunger) which they devour in great quantities. This makes the dissimilarity in the vitamin A concentrations in the liver very striking, as both the specimens were full-grown and were shot in Danish waters in the autumn of 1938. The gannet liver contained about 3500 I.U. per g., while the cormorant contained only about 300. In the polar loom which subsists on nearly the same food, a value of about 1200 I.U. per g. was found.

In the duck group the liver has a fairly constant amount ranging from 150 to 300 I.U. per g., although the garganey, as will be mentioned presently, shows wide deviation.

The kestrel and the merlin differ greatly, although closely related systematically and in their food consumption. The amounts of vitamin A in the two buzzards are high and almost identical, while the shaggy-footed buzzard shows the highest value found in the wild birds examined.

The grouse liver contains about the same amount as those of gannet and polar loom, which shows that birds living on land may take as much vitamin A with their food as marine birds.

The four samples of hen livers are of particular interest, as the animals were of the same kind but had been fed in different ways. Sample I was bought in a shop; sample II is from 5 hens that received 48 % food without vitamin A + 42 % yellow maize from birth till they were killed at 8 weeks of age; sample III is from 5 hens which had received food without vitamin A with an addition of 6 mg. carotene (as carotene oil) per kg. food, and sample IV is from 5 hens that received the vitamin A-free food with the addition of 0.5 % whale liver oil

(containing 190,000 i.u. per g.). Further details of these investigations are given in papers by With [1938] and by With & Wanscher [1939]. As expected, group IV shows a very high concentration of vitamin A in the liver; it is remarkable that the vitamin A in all four cases showed identical physical and chemical properties although it was derived from different sources.

The gulls show fairly high values. Of the passerine birds, the lark has a considerable amount of vitamin A in the liver. The Amazon parrot shows the lowest value found (5–10 i.u. per g.); however, this bird succumbed spontaneously in the Zoo. The golden plover and the currawong were also relatively poor in vitamin A.

The few analyses of reptiles have a certain interest. Of the three lots of vipers examined, the first was a mature specimen killed just before hibernation, the second (a full-grown snake) and the third (two immature snakes) were killed in February, i.e. several months after commencement of the winter sleep. There was no appreciable diminution of vitamin A value in the hibernating reptiles, as the higher value found in the first specimen may be due to the larger size (age) of the animal. Thus probably no considerable amounts of vitamin A are consumed during reptilian hibernation.

General inanition appears to present similarities in this respect, judging from an observation of a specimen of *Varanus komodensis*. This animal had been brought to Denmark from the Comodo Island by Dr Paul Fejos. During the voyage and stay in the Zoo it took practically no nourishment; nevertheless, after its death a very considerable layer of body fat was present and a large amount of vitamin A was found in the liver. As the adipose tissue was extremely poor in vitamin A and carotenoids ($B_{1\text{cm}}^{1\%}$ S 61 = 0.00023; $E_{1\text{cm}}^{1\%}$ S 43 = 0.00032), by far the largest store of vitamin A was contained in the liver. The large store of vitamin A even after about 6 months of inanition is remarkable.

The mammals have shown the same results as the birds. Exceptions are, however, the foxes; one of the specimens (the largest) showed values about 10 times greater than the others.

The whale liver examined was, strangely enough, almost devoid of vitamin A. This animal had been lying for a couple of weeks on the shore, but the temperature was low (winter), a condition which is deemed unfavourable for the destruction of the vitamin. It may thus be assumed that this kind of whale (the grampus) stores insignificant amounts of vitamin A in the liver.

Vitamin A in the mole and the bat—two animals which subsist mainly on insects and worms—has the same physico-chemical properties as vitamin A in the livers of the other animals. Judging from the amount in the bat's liver, insects are rich sources of vitamin A.

The rodents examined have only small amounts in the liver. The capibara examined was a specimen destined for the Zoological Gardens, but it died on the voyage; the body was, however, kept in cold storage until we examined it.

The lack of vitamin A₂ in the otter liver examined has been mentioned above, also the peculiar absorption curves given by the extract of the ermine liver; the concentration in this animal's liver was fairly low. The highest concentration of vitamin A was found in the largest of the foxes (about 2000 i.u. per g. liver); this is about one-third of the highest value in the wild birds.

The domesticated beasts of prey (dog and cat) nearly all have a considerable amount of vitamin A in the liver, although the amount is notably less than in the wild species (fox, fitchet).

The pig (omnivorous) has a rather low value (about 100 i.u. per g.), which is not surprising, as pigs in this country are generally given food poor in vitamin A.

There is considerable variation among the herbivorous animals: the horse and ox show values round about 500 I.U. per g. liver, while the calf (cow II) contains only about 100 I.U., presumably because young animals on the whole have lower reserves of vitamin A than older ones. The sheep (the specimen examined was a lamb) and the roe deer (full-grown animal) show considerably higher values (about 1600 I.U. per g. liver). These differences are not explicable by differences in the fodder, as the horse, cow and sheep receive most of their vitamin A requirements during spring and summer pasturing; the roe deer feeds on plants all the year round and vitamin A in its food is presumably also derived from green buds and leaves in the early summer.

However, the interesting biological problem regarding the variations in vitamin A reserves in the different species has been far from solved by the present material examined; an exhaustive investigation would require analyses of numerous individuals of each species.

The irregularities in the vitamin A contents of the liver have also been found to apply to the carotenoid contents.

Our investigations have led to conclusions which necessitate a revision on some points of the current view of the role of these substances in vitamin A metabolism in the various species. Thus it is stated by several investigators that birds do not store carotene but only phytoanthin, and that carnivora are unable to store and metabolize carotene. It will appear from the present investigations that this is, at any rate, not the case in some species in the groups analysed. Furthermore, it may be mentioned that considerable quantities of carotene are found in the liver of hens shortly after a meal rich in carotene [With & Wanscher, 1939]; this is not necessarily opposed to the results of Palmer *et al.* [Palmer, 1922], in which it was shown that carotene was not stored in the liver of hens under normal conditions. It will be noted that among the birds, the garganey and the currawong have a high concentration of carotenoids in the liver, while the concentration of vitamin A is low. Only in the garganey does the main part of the carotenoids consist of carotene. The high concentration of carotenoid in these animals may be due to a preceding meal rich in carotenoids; though this supposition is rather unlikely, it can be refuted only by analysis of a great number of individuals of these species.

In the carnivora, large amounts of carotene are present in the livers of the badger, fox and fitchet (both the fitchets and all the three foxes examined), the values being of the same order as those found in the horse and the roe deer. Two of the dogs showed a rather high carotene value; these results are perhaps somewhat doubtful as the chromatogram did not give the beautiful β -carotene zone found in the fitchets and foxes.

At first sight, it appears almost absurd that the carotene content of the liver of a decidedly carnivorous animal should be the same as that of herbivora, but a detailed analysis of the carotene content of the food of the former group of animals explains this phenomenon. The fitchet exists mainly on frogs, and the fox similarly devours frogs as well as other lower species. Zechmeister & Tuzson [1936] have shown that the liver of the frog (*Rana esculenta*) contains a considerable amount of carotene, viz. about 10 mg. per kg., besides other carotenoids. Furthermore, carotene is found in moderate amounts in other organs, especially the skin. Even though the carotenoid content of the food of these carnivora is far less than that of the fodder of herbivorous animals, it appears likely that the former animals may consume considerable quantities of carotene, especially as frogs are probably not the only items of their diet containing ample amounts of carotene.

Besides, the fact that the liver of the garganey contains a concentration of carotene 10 times larger than that of the herbivora shows that the storing of carotene in the liver is not proportional to the amount of carotene ingested.

SUMMARY

The amounts of vitamin A and carotenoids in the liver have been determined in 33 different mammals belonging to 21 species, 41 birds of 36 species, 4 reptiles of 2 species and in 8 human specimens.

A detailed spectrographic analysis in the ultraviolet range, a detailed determination of the absorption of the Carr-Price reaction for three different spectral filters and an estimation of the carotenoids, including microchromatography, have been performed on the majority of the liver samples.

The technique employed in the treatment of the material, saponification, extraction, spectrography, measurement of the Carr-Price reaction and the carotenoids is described.

Most livers have yielded typical ultraviolet absorption curves similar to the curve of cod liver oils. Some of the curves are atypical.

Tables I-IV show the extinction coefficient $E_{1\text{ cm}}^{1\%}$ (1 g. liver in 100 ml.; 1 cm. layer thickness) of vitamin A at 328 m μ (column 6), the Carr-Price reaction with the filters S 59, S 61 and S 66.6 in the Pulfrich photometer (columns 3-5) and certain ratios of the extinction values (column 10), a brief description of the absorption curves (columns 8, 9 and 14) and the zoological classification of the species examined (columns 1-2).

It was found that the relation between the extinction of the Carr-Price reaction with S 61 in the Pulfrich photometer and vitamin A absorption at 328 m μ ($B_{1\text{ cm}}^{1\%} \text{ S 61} : E_{1\text{ cm}}^{1\%} \text{ 328 m}\mu$) (column 10) had a fairly constant value of 2.60-2.75, with a coefficient of variation of 10-15 %. (However this only obtains for livers showing a typical absorption curve in the ultraviolet.)

The conversion of the extinctions measured with S 61 in the Carr-Price reaction into I.U. per g. liver must, therefore, be calculated from the formula $B_{1\text{ cm}}^{1\%} \text{ S 61} = 2.60-2.75$, corresponding to about 1600 I.U. per g. (or if the amount of vitamin A is expressed in micrograms by the formula $B_{1\text{ cm}}^{1\%} = 4100-4400$ for the Carr-Price reaction of the pure vitamin A with S 61).

In no instance has vitamin A₂ been demonstrated. The Carr-Price absorption curve has been fairly constant in the livers examined, as illustrated by the variation in the extinction ratios of the reaction with the three spectral filters.

The variation in the amount of vitamin A in the livers of the various species examined was very considerable; the same was the case with the amount of carotenoid (and carotene). There was no simple proportionality between the amount of vitamin A and carotene in the food ingested and the concentration in the liver. Carotene was found in considerable amount in the liver of certain beasts of prey and birds as well as in the herbivora but the majority of the livers examined contained almost no carotenoid.

Our results encourage further investigations on still larger series, comprising a greater number of species and, if possible, a greater number of individuals of each species.

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CCXXI. QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN URINE

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SINCE Warburg & Christian [1934] and von Euler *et al.* [1935] have shown that coferment and cozymase contain nicotinamide, and particularly since the effect of nicotinic acid in the treatment of the disease of blacktongue in dogs [Elvehjem *et al.* 1937] and pellagra in man [Fouts *et al.* 1938] became established, the question of the excretion in the urine of nicotinic acid or its derivatives has attracted considerable attention.

Vilter *et al.* [1938] have devised a colorimetric reaction "for the determination of nicotinic acid, nicotinamide and possibly other pyridine-like substances in human urine" and state: "by the application of this method to human urine it has been found that individuals on a normal diversified diet excrete daily colour-producing substances equivalent to 20 to 50 mg. of nicotinic acid or its conjugates. We believe these substances to be nicotinic acid conjugates."

This method which is based on the colour reaction of pyridine with 2:4-dinitrochlorobenzene and alkali hydroxide [Vongerichten, 1899], is, however, rather intricate and tedious. Moreover, decoloration is performed with charcoal, which seems rather ill-suited for the purpose, since the usual kind of charcoal at any rate adsorbs nicotinic acid [Shaw & Macdonald, 1938; Bandier & Hald, 1939; Porjé, 1939]. Covello [1938] has also employed Vongerichten's method with the modification that he uses 3:4-dinitrochlorobenzene instead of 2:4-dinitrochlorobenzene.

Quite recently, Porjé [1939] has described another colorimetric method for the estimation of nicotinic acid (pyridine derivatives) in urine based on König's [1904] observation that a colour reaction ensues when pyridine reacts with CNBr and a primary or secondary aromatic amine (e.g. aniline). With this method it has been found that normal subjects excrete 1.2 to 20 mg. nicotinic acid in 24 hr. Porjé makes the reservation that the colour reaction developed in analysis of the urine may be due to some other derivative of pyridine, though he does not subject the matter to closer investigation.

In both methods the colour produced by nicotinic acid is very unstable; this makes them unsuitable, at least for serial work. Moreover, Vilter *et al.*, Covello and Porjé perform the analysis without subjecting the urine to hydrolysis, which, as will be shown below, is not without importance.

In this paper, a description will be given of a method for the quantitative estimation of nicotinic acid (—derivative) in urine, involving the colorimetric reaction with CNBr and metol described in a previous article by Bandier & Hald [1939].

As attempts at decoloration (of the urine) with charcoal, fuller's earth and heavy metal salts have been unsuccessful, it has been necessary to introduce a blank value for the original colour of the urine.

If the reaction is performed on untreated urine, added nicotinic acid is not recovered quantitatively. This means that urine contains substances that mask

the colour reaction. Better, although not entirely satisfactory, results are obtained if the reaction is performed in the presence of KH_2PO_4 . However, added nicotinic acid is recovered quantitatively if the urine is first saturated with NaCl and the nicotinic acid extracted with a large excess of acetone; the extract is transferred to water containing KH_2PO_4 , and the colorimetric reaction carried out on this solution.

Saturation with NaCl causes flocculation of the precipitate formed on adding acetone; without NaCl, the precipitate adheres in a slimy layer to the sides of the flask. The necessity of using KH_2PO_4 has been explained in a previous publication [Bandier, 1939]. As some urinary pigment also is extracted by acetone, it is essential to include a blank value for the original colour.

The pH decreases to 2-3 after metol is added. The same value also is obtained by adding about 0.18 ml. 2N H_2SO_4 . On examining a series of urines, it has been found that addition of this amount of H_2SO_4 after treatment with CNBr, has very little influence on the original colour (blank value).

Technique

5 ml. urine and 2 g. NaCl are mixed in a 50 ml. graduated flask with a glass stopper and 45 ml. acetone are added from a burette. The mixture is shaken thoroughly and centrifuged for about 2 min. in a 50 ml. tube capped with thin parchment paper. 20 ml. of the acetone extract (representing 2 ml. urine) are then pipetted off, and placed in a 100 ml. round-bottomed boiling flask together with about 3 ml. distilled water. The acetone extract is evaporated *in vacuo* with continual shaking (bath temperature 40-50°).

After evaporation of the acetone, the contents are transferred quantitatively with the aid of 5 ml. 2% KH_2PO_4 to a graduated 20 ml. flask and heated for 5 min. on a water bath at 75-80°. Then 1 ml. 4% freshly prepared aqueous CNBr is added, and after 5 min. on the water bath the flask is cooled under the tap to about room temperature. 10 ml. of a fresh aqueous saturated solution of metol (about 5%) are added and the total volume made up to 20 ml. with distilled water. After 1 hr. at room temperature, protected from light, the colour developed is read off in a Pulfrich photometer (filter S 43) against a solution prepared simultaneously, containing the same amounts of CNBr, metol, KH_2PO_4 and distilled water to make up the volume to 20 ml.

For the blank, a second portion of 20 ml. of the acetone extract is treated as above, 0.18 ml. 2N H_2SO_4 being added instead of metol. The colour is measured in the Pulfrich photometer (filter S 43) with distilled water in the other cell. The value found is reduced to correspond to the same thickness of cell as used in the first reading and subtracted from this. From the result and the coefficient of extinction determined on a standard solution (0.1 mg. nicotinic acid) the amount of nicotinic acid corresponding to the colour developed can be calculated. The blank value for untreated normal urine may amount to 6 times the value of the colour reaction proper; for urine subjected to alkaline hydrolysis, the blank value is about twice the value of the colour reaction.

As the method is based on a reaction that is characteristic of pyridine, the intensity of the colour cannot be accepted directly as a quantitative measure of nicotinic acid. Thus besides free nicotinic acid, the following pyridine derivatives at least are liable to be encountered: nicotinamide—free and in combination in cozymase and coenzyme [Warburg & Christian, 1936], nicotine (demonstrated in the urine of tobacco smokers by Helmer *et al.* [1939], methylpyridinium hydroxide [Kutscher & Lohmann, 1906] and trigonelline [Linneweh & Reinwein, 1932]. In this connexion it should be mentioned that Ackermann

[1912] found nicotinuric acid in the urine of a dog that had received nicotinic acid by mouth.

Methylpyridinium hydroxide and trigonelline give no coloration, nor presumably do coenzyme and cozymase, as the pyridine N atom is pentavalent in the two latter [von Euler & Schlenk, 1937] as well as in the two former substances. Moreover, it is stated by Warburg & Christian [1935] that active coferment yields no colour with CNBr and sulphanilic acid. Nicotine, nicotinamide and nicotinuric acid give the same yellow colour as nicotinic acid. In equimolar concentrations, the colour produced by nicotinamide is considerably stronger than that yielded by nicotinic acid, while nicotinuric acid, and particularly nicotine, cause feebler colorations. The relations are the following: nicotinic acid: 100; nicotinamide: 142; nicotinuric acid: 42; nicotine: 8.

A desirable first step in the analysis would be, therefore, to transform the derivatives into nicotinic acid. Coenzyme, cozymase and nicotinamide are easily hydrolysed by heating with strong (about *N*) acid or alkali. Nicotinuric acid, however, is uninfluenced by heating with about *N* HCl up to 1 hr. on a boiling water bath, but is easily decomposed in alkaline solution. Thus heating on a boiling water bath for 15–30 min. with *N* NaOH causes complete hydrolysis and liberation of nicotinic acid. (The investigations have been carried out on pure nicotinuric acid, synthetically prepared in this laboratory by Jens Hald.)

Trigonelline and methylpyridinium hydroxide are not affected by heating on a water bath with strong acid or alkali.

As a result of these findings, the analysis has been performed as follows: 15 ml. urine and 2 ml. 10*N* NaOH are mixed in a 20 ml. graduated flask which is closed with a wad of non-absorbent cotton wool and heated for 30 min. on a boiling water bath (which is sufficient to produce a maximal colour reaction subsequently). After cooling the solution, the *pH* is adjusted to about 5 by adding 36 % HCl (usually 1.6 ml. HCl), and the volume made up to 20 ml. with distilled water (the *pH* is determined with narrow strips of "Universalindicator-papier Merck"). After centrifuging, the analysis proceeds as described above. It follows that in calculating the amount of nicotinic acid, the degree of dilution of the urine must be taken into account.

By this procedure, the total amount of free and conjugated nicotinic acid is determined. Nicotine is also included, while methylpyridinium hydroxide and trigonelline give no colour. Similarly, investigations of the specificity of colorimetric reaction have shown that no colour is yielded by picoline or picolinic acid. Thus it seems that pyridine derivatives in which the pyridine nitrogen is pentavalent, and α -substituted derivatives are inactive in the colour reaction. When the analysis is performed with KH_2PO_4 , small amounts of pyridine yield no colour; large quantities (about 1 mg.), which cause the solution to smell strongly of pyridine, give a pale yellow colour which is inconstant and unstable. If KH_2PO_4 is not used, the colour developed is stronger, but it is still unstable and inconstant.

The colour developed is stronger when the urine is hydrolysed with HCl than it is after alkaline treatment; this enhanced colour reaction is not however due to nicotinic acid.

15 ml. urine are heated with 1.6 ml. conc. HCl on a boiling water bath for about 30 min. and the *pH* adjusted to about 5 with 2 ml. 10*N* NaOH. The analysis then proceeds as described above, partly with KH_2PO_4 and partly with 1 ml. 36 % HCl in the main analyses as well as in the blanks. The addition of HCl eliminates nicotinic acid from the colour reaction, as this substance yields no colour with CNBr and metol in the presence of free strong acid. Concurrently,

a second sample of urine is subjected to alkaline hydrolysis and processed partly with KH_2PO_4 and partly with HCl .

On carrying out the colour reaction after HCl has been added, it is advisable to heat and cool the main analysis and the blank together, as the remainder of the original colour of the urine turns darker on being heated with HCl and CNBr .

The results are shown in Table I.

Table I

	Colour-yielding substances equivalent to nicotinic acid, mg. per 100 ml.
1. Analysis of urine after acid hydrolysis and with the use of 5 ml. 2% KH_2PO_4	0.58
2. After acid hydrolysis and with the use of 1 ml. conc. 36% HCl	0.35
Difference between 1 and 2	0.23
3. Analysis of urine after alkaline hydrolysis and with the use of 5 ml. 2% KH_2PO_4	0.24
4. After alkaline hydrolysis and with the use of 1 ml. conc. HCl	0.00

It will be noticed that the increased power to produce colour induced by subjecting the urine to acid hydrolysis, is maintained when the analysis is performed in the presence of HCl (where nicotinic acid, as already mentioned, yields no colour).

The difference between the colour reactions of urine subjected to acid hydrolysis at the usual reaction and in a strongly acid solution, corresponds fairly well to the strength of the colour yielded by the sample of urine subjected to alkaline hydrolysis. In this sample, as in the case of pure nicotinic acid, the colour fails to appear in the presence of HCl .

RESULTS

Analyses of a series of daily urines from healthy adults on a normal mixed diet containing an adequate supply of meat have given the results contained in Table II.

Table II. *Nicotinic acid excretion by normal subjects on mixed diet*

No.	Diuresis ml.	Colour-yielding matter equivalent to nicotinic acid, mg. per 100 ml.		Total excretion of colour-yielding substances equivalent to nicotinic acid (after alkaline hydrolysis), mg.	Urine + 0.67 mg. nicotinic acid per 100 ml. Total nicotinic acid found (after alkaline hydrolysis), mg. per 100 ml.	Nicotinic acid recovered %
		Urine not subjected to hydrolysis	Urine subjected to alkaline hydrolysis			
1	850	0.14	0.23	2.0	0.89	98
2	830	0.09	0.29	2.4	0.93	96
3	1565	0.15	0.32	5.0	1.01	103
4	725	0.07	0.21	1.5	0.88	100
5	1430	—	0.27	3.9	0.92	97
6	1100	—	0.30	3.3	0.98	101
7	1550	—	0.23	3.6	0.91	101
8	1150	—	0.20	2.3	0.88	101
9	780	0.09	0.30	2.3	0.96	98
10	1070	—	0.37	4.0	1.05	102

In order to investigate the mode of excretion in healthy adults of pure nicotinic acid ingested by mouth, the following experiment was performed.

After securing a specimen of urine from the preceding 24 hr., 90 mg. nicotinic acid (3 tablets of 30 mg. taken with a large glass of water) were given on an empty stomach, and the urine collected first at hourly intervals and subsequently at intervals of several hours. An example is shown in Fig. 1.

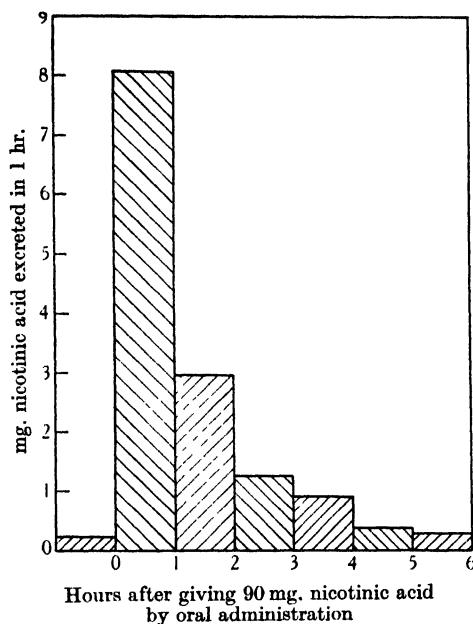


Fig. 1. Diagram showing the excretion in the urine of nicotinic acid given by mouth. Analysis carried out on alkaline-hydrolysed urine.

The calculated amounts of nicotinic acid were recovered from urine subjected to alkaline hydrolysis. The excretion is largest during the first hour, but rapidly diminishes to the normal level in about 6 hr. In the case illustrated, 13.75 mg. nicotinic acid were excreted during the first 6 hr.; subtraction of the normal amount contained in the urine (determined by analysis of the urine passed before the ingestion of nicotinic acid), i.e. 1.25 mg., gives 12.5 mg.; this is 13.9 % of the ingested amount.

On considering the results given in Table III of a series of analyses of urine passed by a normal person during the first hour after ingestion of pure nicotinic

Table III. *Analyses of urine passed by a normal subject after ingestion of nicotinic acid*

	Colour-producing substances equivalent to nicotinic acid in mg. per 100 ml.
Analysis of urine not subjected to hydrolysis	3.30
Analysis of urine subjected to acid hydrolysis	3.58
Analysis of urine heated with NaOH on a boiling water bath for 15 min.	6.02
Analysis of urine heated with NaOH on a boiling water bath for 30 min.	6.11
Analysis of urine heated with NaOH on a boiling water bath for 60 min.	6.09

acid by mouth, it seems probable that most of the nicotinic acid recovered from the urine was excreted as nicotinuric acid. It has been mentioned already, that this substance promptly liberates nicotinic acid on being subjected to alkaline hydrolysis, while it is not attacked by heating in the presence of acid under the given conditions. Computed for equivalent amounts, the coefficient of extinction of nicotinuric acid is $2/5$ of the coefficient of extinction of nicotinic acid.

Urine contains substances which mask the colour reaction; the masking is most conspicuous when a large amount of nicotinic acid is present, but is relatively insignificant for amounts approaching values in normal urines. This drawback has been obviated in the present method of analysis by extracting with acetone.

It is imperative, therefore, to perform the extraction with acetone in analysis of urine containing a large amount of nicotinic acid (e.g. in investigations on the excretion of nicotinic acid after ingestion of a certain amount). However, it is possible that the extraction may be omitted in analyses of urines from ordinary patients; the colour reaction could then be performed in the presence of KH_2PO_4 directly on the urine after alkaline hydrolysis. Nevertheless, the simplified method should not be used before reliable data on the variations in urines from normal persons are available.

SUMMARY

1. An account is given of a technique for the quantitative estimation in urine of nicotinic acid and nicotinic acid conjugates by the colorimetric method involving the use of CNBr and metol described in a previous paper.

2. Part of the nicotinic acid or its conjugates must be present in the urine in combined form, as the reaction increases two- or three-fold if the urine is first subjected to alkaline hydrolysis.

3. Acid hydrolysis of the urine liberates substances that yield a similar colour reaction which cannot however be due to nicotinic acid.

4. The urines of 10 normal persons have been analysed after alkaline hydrolysis; it was found that the colour-producing substances excreted in 24 hr. were equivalent to 1.5–5 mg. nicotinic acid.

5. After oral ingestion of 90 mg. nicotinic acid, 14% is excreted, chiefly during the first hour.

6. After oral ingestion, part of the nicotinic acid is excreted in a combined form which is hydrolysed under the same conditions as is synthetic nicotinuric acid (nicotinyl glycine).

I wish to thank the P. Carl Petersen Foundation for a grant which has covered part of the expenses of this investigation.

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Note added 1 November 1939. Further study has shown that in investigations on urines from ordinary patients, i.e. *without previous ingestion of free nicotinic acid or nicotinamide*, the colour reaction can be performed on 2 ml. of alkaline-hydrolysed urine after the addition of 5 ml. 2% KH_2PO_4 .

For the blank another portion of 2 ml. of the alkaline-hydrolysed urine is treated in the same way, 0.18 ml. 2*N* H_2SO_4 being added instead of metol.

Care must be taken that the patients do not smoke tobacco, as nicotine, like coramine (diethylnicotinamide) will give a slight colour reaction with CNBr and metol.

CCXXII. METABOLISM OF SULPHUR

X. THE REPLACEABILITY OF *dl*-METHIONINE IN THE DIET OF ALBINO RATS WITH ITS PARTIALLY OXIDIZED DERIVATIVE, *dl*-METHIONINE SULPHOXIDE¹

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(Received 5 August 1939)

IN view of the fact that methionine has been found necessary for growth its derivatives become of biological importance. An oxidation product, *dl*-methionine sulphoxide, has been prepared [Toennies, 1938; Toennies & Kolb, 1939], which should be of interest as a possible intermediate oxide of sulphur in metabolism. The roles of several oxides of *l*-cystine in the intermediary metabolism of cystine have been previously investigated [Bennett, 1937; 1939]. Growth curves were obtained on albino rats fed *l*-cystine disulphoxide, *l*-cysteinesulphinic acid and *S*-(guanyltio)-cysteine.2HCl, which hydrolyses to *l*-cysteinesulphonic acid. *l*-cystine disulphoxide proved capable of replacing *l*-cystine in the diet, but only one molecule of cystine is apparently available from three of the disulphoxide. The *l*-cysteinesulphinic acid produced no growth, while *l*-cysteinesulphonic acid gave rise to a slight but definite increase in growth. The present experiment was undertaken to find out whether *dl*-methionine sulphoxide could replace the growth-promoting properties of *dl*-methionine in the diet of the albino rat.

Preparation of compounds

The compounds employed were: *dl*-methionine (Eastman), 98.0 % purity according to S determination; *dl*-methionine sulphoxide [Toennies & Kolb, 1939], 99.8–99.9 % purity (0.05 % cystine); arachin isolated from peanut meal² by the method of Johns & Jones [1916]. Acetone was used as the drying agent instead of alcohol and ether. The arachin used in the present experiment was analysed for methionine by the method of Kassel & Brand [1938].³ The volatile iodide determinations gave a higher figure for methionine than the homocysteine titration, the values according to the former being 0.93 % and to the latter 0.53 % of the undried protein; the cystine values were 0.47 % if the higher methionine value be accepted, and 0.81 % if the lower be taken.⁴ Organic drying agents such as acetone and alcohol-ether, do not seem to be the cause

¹ Aided by a grant from the Robert McNeil Fellowship.

² I wish to thank the Planters Peanut Co. for the peanut meal.

³ I am indebted to Mr Thomas P. Callan for these determinations.

⁴ Eight determinations of methionine, done on three other dried preparations, gave an average of $0.79\% \pm 0.12$ by the volatile iodide determinations and $0.47\% \pm 0.04$ by the homocysteine titration. At present the cause of this variation found in arachin is not known.

of the discrepancy since substantially the same results were obtained on acetone-, alcohol-ether- and air-dried samples. The arachin used when dried to constant weight at 100° lost approximately 7.5%, and had 0.86% ash. The value of 0.77% methionine given for arachin in a previous paper [Bennett, 1939] was based on the volatile iodide figure, the homocysteine titration being 0.38%.

Animal experiments

Albino rats, Wistar strain, were used as experimental animals. They were kept on a methionine-deficient basal diet, a modification of the cystine-deficient diet of Dyer & du Vigneaud [1936], 16% milk vitamin concentrate, 15% arachin as the basal protein and 24% dextrin; each rat received 100 mg. of Harris vitamin B complex daily. Sufficient dry mixture was made at the beginning of the experiment to last through the entire period. From this, fresh basal diet was prepared every 3 days. All food was kept in the refrigerator. The special compounds were fed individually in small pieces of butter, the control animals receiving the same amount of butter. These butter mixtures were made once a week, keeping all instruments on ice during the mixing, and were kept in individual covered glass dishes in the refrigerator at 0°, the methionine sulphoxide at -10°. The animals were maintained on a normal diet for 8 days, at the end of which the basal diet was supplemented with the special compounds for a period of 19 days. The rats were weighed every other day and the average weight of the group plotted. The approximate amount of the basal diet consumed per rat each day was determined by weighing daily the basal food given each group and the residual food and dividing by the number of animals in the group (Table I).

Table I. *Daily basal food consumption per rat in g. averaged over 2-day period*

Days	Group A	Group B	Group C
	Unsupplemented		
1-2	5.8	4.7	4.7
2-4	6.7	5.9	5.9
4-6	7.9	7.1	7.1
6-8	7.5	6.9	6.9
	Daily supplement		
	Unsupplemented control	<i>dl</i> -methionine mg. 6.00	<i>dl</i> -methionine sulphoxide, mg. 6.64
8-10	5.8	5.3	5.4
10-12	5.3	4.9	5.5
12-14	6.5	5.8	6.2
14-16	5.5	5.3	5.9
16-18	5.5	6.2	6.1
18-20	5.5	6.0	6.2
20-22	5.7	6.3	7.0
22-24	6.0	7.1	6.7
24-26	5.9	6.9	6.8
27	6.5	7.6	6.8

In the experiment reported, 9 male rats from two litters born on the same day, were divided into three comparable groups of three animals each, 25 days old. After 8 days on the methionine-deficient diet, group B was supplemented with 6 mg. *dl*-methionine daily for 19 additional days, group C with 6.64 mg.

dl-methionine sulphoxide, the S equivalent of 6 mg. *dl*-methionine, and group A was unsupplemented. Fig. 1 shows the results obtained; the curves for groups B and C practically coincide.

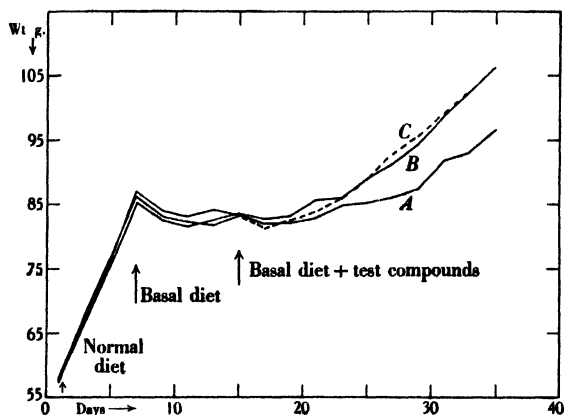


Fig. 1. Growth curves expressing the quantitative metabolic relationship of *dl*-methionine and *dl*-methionine sulphoxide. The casein of du Vigneaud's basal diet was replaced by arachin. A, control; B, *dl*-methionine; C, *dl*-methionine sulphoxide.

DISCUSSION

The sulphoxide of methionine, according to Toennies & Kolb [1939], has certain properties, such as increased solubility, in common with the corresponding cystine derivative, cystine disulphoxide, because of the state of oxidation of the sulphur. Yet these authors state that the oxygen potential of the methionine sulphoxide, to judge from its behaviour *in vitro* with iodide and cysteine, is much lower than that of the cystine derivative. There is an "absence of any tendency of the methionine sulphoxide to form sulphenic or sulphinic acids by hydrolytic cleavage which is so pronounced in the case of cystine disulphoxide and characteristic of the —S—S— linkage". However, in the present experiment, the *dl*-methionine sulphoxide proved capable of replacing *dl*-methionine; identical growth curves were obtained with both compounds. It would seem from these results that *dl*-methionine sulphoxide could possibly play an intermediate role in the metabolism of *dl*-methionine.

An arachin basal diet seems to produce growth in the control groups. Experiments reported by White & Beach [1937] and Baernstein [1938] show an upward trend of their control curves. Baernstein states that 9 rats fed a 20% arachin diet showed very poor growth and lost weight at first but soon regained their original weight. "A slow growth ensued which averaged 0.5 g. per day." In the present experiment the rats in the control group lost weight at first, but in 22 days they had come back to their original weight and continued to gain weight until the end of the experiment. In spite of the growth attained by the controls, the animals on the supplemented diet consumed a greater amount of methionine, since their daily consumption of the basal diet was practically the same. Group A ate an average of 5.8 g. per rat each day, group B, 6.1 g. and group C, 6.2 g. Because of the discrepancies in the methionine values of the arachin (see preparation of compounds), it is difficult to state the exact amount of methionine in the basal diet. The homocysteine titration figures agree quite well with the original analysis by Baernstein [1932]. Some unknown impurity may be the

cause of the high volatile iodide figures. If the homocysteine titrations are correct, the basal diet would have 0.08% methionine and 0.12% cystine; if the volatile iodide figures are correct, the basal diet would contain 0.14% methionine and 0.07% cystine.

SUMMARY

Albino rats showed identical growth curves when *dl*-methionine and *dl*-methionine sulphoxide, respectively, were added to a methionine-deficient diet. Therefore under the conditions of the experiment reported, *dl*-methionine sulphoxide is able to replace *dl*-methionine in the diet of the albino rat.

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CCXXIII. *IN VITRO* ACTION OF INSULIN ON MINCED AVIAN AND MAMMALIAN MUSCLE¹

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(Received 31 August 1939)

IN 1938 Krebs & Eggleston reported the interesting observation of an *in vitro* insulin effect upon oxidative metabolism. The experimental conditions which they employed to demonstrate this phenomenon consisted in the use of minced pigeon muscle as against the tissue slice technique which has been uniformly unsuccessful for the demonstration of insulin effects on respiration. A *M*/10 phosphate (*pH* 6.8) buffer containing the minced tissue in at most a one to ten dilution, the addition of citrate, and finally of boiled muscle juice resulted in a system which gave maximum results. With this system, increases of respiration of as much as 90% were noted in the presence of insulin. These effects were obtained with an amorphous insulin hydrochloride preparation (B.D.H.), but were absent when crystalline insulin was employed. This failure with crystalline insulin was attributed to its zinc content, an interpretation based on the inhibiting effects of zinc on the respiration of pigeon mince. The stimulation of respiration was roughly proportional to the insulin concentration between 0.05 and 5.0 mg./100 ml. Instead of citrate, other members of the postulated citric acid cycle could be substituted with similar effects. The only tissue employed in these experiments was the breast muscle of the pigeon, which was minced in a Latapie mincer.

From these experiments, the authors drew several conclusions. From the catalytic effects of certain dicarboxylic acids on the respiration of minced pigeon breast, Krebs & Johnson had previously proposed [1937] that they constituted a "citric acid cycle" which was responsible for the maintenance of carbohydrate metabolism. Since the citrate effect on respiration was now found to be increased in the presence of insulin, they concluded that the hormone influenced the metabolism of carbohydrate by acting catalytically on the citric acid cycle. They regarded the crystalline zinc insulin as either an unnatural form of the hormone or as an inactive depot form.

Because of the potential importance of these observations in elucidating the relationship of insulin to sugar metabolism, it was thought worth while to examine a variety of experimental animals to determine how general this phenomenon was. In order to insure the reliability of the procedures employed in this study, they were controlled by a repetition of much of the work reported by Krebs & Eggleston with pigeon breast muscle. In general, their results were confirmed. Thereafter, comparable studies were extended to the minced skeletal muscles of the chicken, dog, cat and rabbit, and the cardiac muscle of the dog. With none of these tissues was a similar insulin effect noted.

¹ This investigation was supported by grants from the Committee on Research in Endocrinology of the National Research Council, and from the Carnegie Corporation. A preliminary report was presented at the Toronto meeting of the Federation of American Societies for Experimental Biology [Shorr & Barker, 1939].

METHODS

The pigeons were guillotined, the breast muscle rapidly plucked and immediately chilled. The same technique and the same muscle was employed with the chicken. Skeletal muscle was obtained from cats and dogs from the hamstrings with the animals under nembutal anaesthesia. Cardiac muscle was excised under the same conditions. Rabbit muscle was obtained after the animal was stunned by a blow. All tissue was rapidly chilled on ice and ground in a cold Latapie mincer. The mince obtained was thoroughly mixed, portions weighed out, suspended in the chilled media and pipetted into the conventional Warburg vessel with alkali in the wells for O_2 consumption measurements. All determinations were made in triplicate in pure O_2 at a temperature of 40° for the pigeon and 37.5° for the mammalian muscle.

The solutions used duplicated those of Krebs & Eggleston:

- (1) $M/10$ sodium phosphate, pH 6.8.
- (2) Boiled muscle extract prepared by extracting finely minced fresh beef heart with an equal volume of water for 10 min. in a boiling water bath and filtering.
- (3) $0.2 M$ trisodium citrate.
- (4) Insulin solutions containing, per 0.2 ml., 0.001–5.00 mg. of B.D.H. insulin hydrochloride or Lilly amorphous insulin. The Lilly insulin, which was furnished through the kindness of Dr G. H. A. Clowes, had an activity of approximately 22 units per mg. The final concentration of insulin in the suspensions ranged from 0.05 to 75 mg./100 ml.

The phosphate solution was used alone, and in combination with muscle extract and citrate in the proportions of 30:10:3 respectively. In some of the mammalian experiments the phosphate concentration used to make up the combinations was increased to $M/7$ in order to yield a final phosphate concentration of $M/10$.

In some experiments comparison of the respiratory metabolism of the mince was made with unminced dog skeletal muscle. For this purpose fibre bundles of suitable diameter were prepared from dog skeletal muscle by careful dissection, as previously described [Richardson *et al.* 1930]. This method yields a uniform preparation of intact fibres. Pigeon muscle was found too delicate to permit a satisfactory dissection of muscle bundles in this manner; therefore, slicing was resorted to. Cellular destruction was minimized in this case by cutting the sections parallel to the direction of the fibres after first dissecting out a layer of muscle with a uniform arrangement of fibres. By this means a muscle strip was obtained with a high percentage of relatively intact muscle fibres, as judged from histological sections. While not quite as satisfactory from the standpoint of lack of damage as the dog muscle strips, these slices respire for long periods of time at a very uniform rate. With these two unminced tissue preparations, the phosphate content of the medium was reduced to $M/75$, the conventional Ringer solution employed, and the pH raised to 7.4 in accordance with the experience of this laboratory as to conditions for optimal respiration of muscle.

The duration of the experiments and the amounts of tissue employed were varied in an effort to obtain the maximum effects of the components used. For convenience in comparing results, the respiration in all experiments shown in the Tables has been calculated on the basis of $\mu l. O_2$ per mg. wet weight of tissue per hr. In most of the pigeon experiments, 200–300 mg. of the mince were used per vessel, and the duration of the experiments was from 3 to 5 hr. Approximately the same amounts of tissue were employed in the mammalian series, but here respiration usually fell off more rapidly and the experiments were terminated in 2 to 4 hr.

The in vitro effects of insulin on the respiration of minced pigeon muscle

In the main, the results obtained by Krebs & Eggleston were confirmed. Citrate, muscle juice and insulin each exerted an additional stimulation of the respiration of the minced muscle in phosphate alone. Fig. 1, a typical experiment, shows a definite superiority of citrate plus phosphate over phosphate alone, and of muscle juice plus citrate plus phosphate over citrate plus phosphate. Finally, the addition of insulin caused a further increase in O_2 consumption amounting in this instance to 27 %. The improvement in respiration produced by

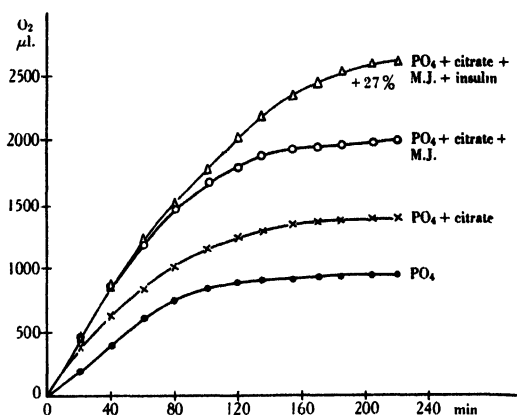


Fig. 1. Effects of citrate, muscle extract, and insulin on O_2 consumption of minced pigeon breast muscle.

Table I. *Effect of insulin on respiration of minced pigeon breast muscle*

Condition of animal	Duration of exp. hr.	Q_{O_2}		Increase due to insulin %	Amount and kind of insulin mg./ml.
		No insulin $\mu l./mg./hr.$	Insulin $\mu l./mg./hr.$		
Fed	3.7	2.99	3.81	27	5, B.D.H.
,,	3.5	2.76	3.40	23	5, Lilly
			3.27	19	5, B.D.H.
Fasted	4.5	2.88	3.27	14	5, B.D.H.
Fed	3.0	5.45	5.87	8	5, B.D.H.
,,	3.2	2.47	3.12	26	4, B.D.H.
			5.12	18	6, Lilly
			3.50	8	10, B.D.H.
,,	2.4	3.25			
			4.09	20	7.5, B.D.H.
			4.40	20	7.5, B.D.H.
,,	3.3	3.61			
			4.54	22	7.5, B.D.H.
			4.59	24	7.5, B.D.H.
Fasted	2.7	2.76	3.18	15	7.5, B.D.H.
Fed	3.4	1.40	1.78	27	1.5, B.D.H.
			2.14	53	6, B.D.H.
			2.10	50	30, B.D.H.
			2.09	49	60, B.D.H.
Fed	5.5	2.26	2.28	1	0.05, B.D.H.
			2.34	3	0.5, B.D.H.
			2.79	23	5.0, B.D.H.
,,	4.3	2.97	3.52	19	5.0, Lilly
			3.55	19	5.0, Lilly
			3.14	26	5.0, Lilly
,,	3.3	2.59	3.23	25	5.0, Lilly

each additional component was manifested both by an increase and better maintenance of O_2 consumption.

Table I is a summary of the experiments with pigeon breast mince. They show, uniformly, a better respiration when insulin was added to the phosphate-muscle extract-citrate-mince system. B.D.H. insulin hydrochloride and Lilly amorphous insulin were equally effective. The average improvement in respiration was about 25%, considerably less than the values of 40–90% shown by Krebs & Eggleston. The stimulation was minimal with less than about 1 mg./100 ml. of insulin, but, above this level, did not seem any better at 4, or 75 mg./100 ml. In the experiment showing the most pronounced insulin effect, a 50% increase in oxygen consumption was obtained with 6 mg./100 ml. Raising the concentration to 30 and 60 mg./100 ml. had no further effect. Fig. 2 details an experiment with two insulin concentrations below 1 mg./100 ml. and one above.

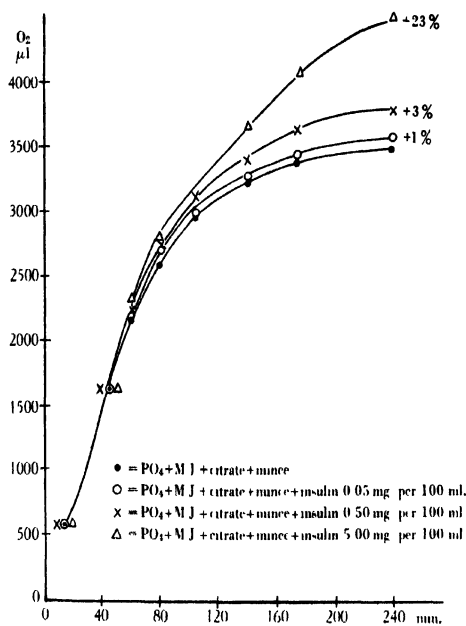


Fig. 2. Effect of increasing quantities of insulin on O_2 uptake of minced pigeon breast muscle.

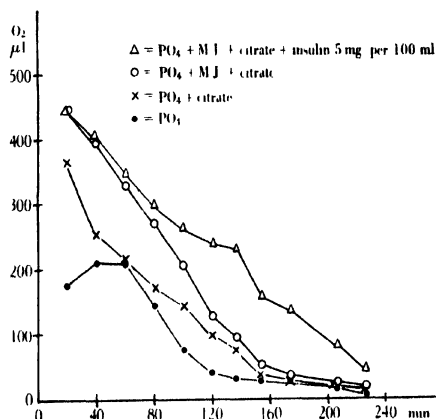


Fig. 3. Increments of respiration of minced pigeon breast muscle.

The insulin effect has been described as a better maintenance of respiration. An inspection of a curve of successive increments in a typical experiment (Fig. 3), makes somewhat clearer the nature of the increased respiration in the presence of insulin. It is evident that whether or not insulin is added, the O_2 consumption of the mince falls off very rapidly. By 100 min., the rate of O_2 consumption is about half of the initial. From then on, the effect of insulin is apparent in delaying the fall of respiration in a system whose rate of O_2 consumption is diminishing rapidly. It would seem more accurate, therefore, to regard an increased O_2 consumption in the presence of insulin neither as a stimulation nor as a maintenance of respiration, but rather as due to a lag in the rate at which it is falling off.

An explanation for the effect of insulin in a mince system, in contrast to the absence of similar effect in slices, might be that destruction of the cell eliminated

the factor of impermeability to the large insulin molecule, and brought about conditions under which it had easy access to the reacting systems. Even with pigeon breast muscle, disintegration of the tissue is essential for the production of the insulin effect. This can be seen from experiments with sliced pigeon muscle (Table II), in which no insulin effect is shown.

Table II. *Effect of insulin on respiration of sliced pigeon muscle*

Ringer + <i>M</i> /75 phosphate + citrate	Q_{O_2} 0.59
" " + insulin, 6 mg./100 ml.	0.51
Ringer + phosphate + glucose	0.29
" " + insulin, 6 mg./100 ml.	0.29

Table III. *Effect of insulin on respiration of minced chicken muscle*

Insulin mg./100 ml. ...	Q_{O_2} Phosphate + muscle extract + citrate			
	0	0.5	5.0	25.0
1.11		0.99	1.07	1.06
0.59		0.57	0.59	0.55

The experiments reported with pigeon breast muscle are in general agreement with those of Krebs & Eggleston, differing chiefly in the extent of the insulin effect. The extension of the study to a variety of other tissues, using the same technique, showed a uniform absence of this phenomenon.

Insulin effect on chicken breast mince. Entirely negative results were obtained with the minced breast muscle of the chicken. Table III summarizes the data in two such experiments. This result was surprising in view of the close relationship of these two experimental animals.

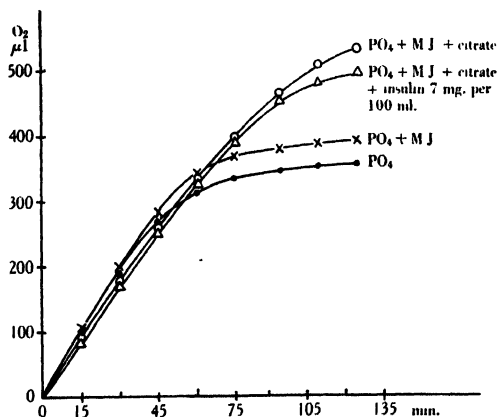


Fig. 4. Effects of citrate, muscle extract, and insulin on O_2 consumption of minced mammalian skeletal muscle (dog).

In vitro effects of insulin on the respiration of minced mammalian muscle

Addition of citrate and muscle juice to the basic phosphate suspension of minced skeletal muscle from the dog, cat and rabbit, and dog cardiac tissue, produced increases in respiration comparable with those obtained with the pigeon mince. When insulin was added no further rise was obtained. Fig. 4 illustrates

such an experiment. The addition of muscle juice to the phosphate solution increased respiration by 10 %. The further addition of citrate to the phosphate and muscle juice raised respiration by 50 % over phosphate alone, while insulin added to the combination actually caused a fall of 10 %.

Table IV. *Effect of insulin on respiration of minced mammalian muscle*

Muscle	Duration of exp. hr.	Q_{O_2}		Amount and kind of insulin mg./100 ml.
		No insulin μ l./mg./hr.	Insulin μ l./mg./hr.	
Dog skeletal:				
Post-absorptive	3.3	0.71	0.69 0.67 0.56 0.53	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	2.1	0.89	0.83	7, B.D.H.
Phlorhizinized-fasted	3.3	0.81	0.74	6.5, B.D.H.
Post-absorptive	3.5	0.60	0.60 0.58 0.56	0.4, B.D.H. 2, B.D.H. 8, B.D.H.
Depancreatized-fasted	2.3	1.01	0.95	3, B.D.H.
Cat skeletal:				
Post-absorptive	3.8	1.45	1.42	6, Lilly
	3.2	1.03	0.82	6, Lilly
	3.9	0.95	0.80 0.91 0.88 0.83	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	3.7	0.90	0.94 0.84 0.83 0.86	1.5, B.D.H. 6, B.D.H. 30, B.D.H. 60, B.D.H.
	3.7	1.17	1.17 1.13 1.20 1.12	1.5, Lilly 6, Lilly 30, Lilly 60, Lilly
Rabbit skeletal:				
Post-absorptive	2.3	0.60	0.56 0.59 0.58 0.59	1.5, Lilly 6, Lilly 30, Lilly 60, Lilly
Dog cardiac:				
Phlorhizinized-fasted	2.6	1.35	1.16	8, B.D.H.
	3.5	1.23	1.28 1.22 1.16	0.3, B.D.H. 1.5, B.D.H. 6, B.D.H.
Post-absorptive	3.4	1.10	1.08 0.94 0.93	0.4, B.D.H. 2, B.D.H. 8, B.D.H.
Fasted	4.2	0.91	0.92 0.87 0.94	0.5, B.D.H. 2.5, B.D.H. 10, B.D.H.
	2.6	1.00	1.07 1.03 1.17	0.05, B.D.H. 0.5, B.D.H. 5, B.D.H.
Depancreatized-fasted	2.5	1.21	1.14 1.13 1.09	0.05, B.D.H. 0.5, B.D.H. 5.0, B.D.H.

The experiments performed on this group of mammalian tissues are summarized in Table IV. No stimulating effect on respiration was noted from insulin, in concentrations ranging from 0.5 to 60 mg./100 ml. In fact, depressions of considerable magnitude were often encountered, both with B.D.H. insulin hydrochloride, as well as the Lilly amorphous insulin. It was thought that this depressant effect might be due to the zinc present in the insulin preparations. Krebs & Eggleston found respiration lowered by 22% when sufficient zinc was added to the pigeon muscle mince-insulin system to make a final concentration of 10^{-6} *M*. According to information from Dr Clowes, the Lilly insulin furnished us contained less than 0.025% of zinc. Since our decreased Q_{O_2} were often obtained with amounts of insulin less than 5 mg./100 ml. the effective zinc concentration required to depress respiration in mammalian muscles would be less than 2×10^{-7} *M*. Actual testing of the effect of added zinc shows that mammalian muscle mince is even more resistant to this metal than pigeon mince (Table V). Thus, the decreases in oxygen consumption were more likely due to the insulin itself.

Table V. *Effect of zinc upon respiration of minced cat skeletal muscle*

Zinc concentration, <i>M</i>	0	10^{-5}	10^{-6}	10^{-7}
Q_{O_2}	0.33	0.32	0.33	0.32
Inhibition Q_{O_2} , no insulin	—	30	11 [Krebs & Eggleston, 1938]	—
Inhibition Q_{O_2} , insulin	—	38	22	—

Table VI. *Effects of zinc, amorphous insulin and crystalline zinc insulin on minced pigeon muscle*

Exp. no.	No additions	Q_{O_2}			
		Zinc 5×10^{-6} <i>M</i>	Amorphous insulin 5 mg./100 ml.	Amorphous insulin + zinc	Crystalline zinc insulin 5 mg./100 ml.
1	2.97	—	3.52	—	3.47
2	3.55	—	4.21	—	4.04
3	2.49	1.98	3.14	2.42	3.53
4	2.59	2.26	3.23	2.71	3.18

Because of the discrepancy, noted above in the effects of zinc on O_2 consumption, experiments were performed on minced pigeon muscle with added zinc, zinc plus amorphous insulin, and crystalline zinc insulin. The amount of zinc added was about the same as that actually present in the crystalline insulin. The results, in Table VI, reveal that added zinc depresses respiration of pigeon muscle, in confirmation of Krebs & Eggleston, and that insulin is able to offset this effect, at least to some extent. The most interesting fact, however, is that when the zinc is actually in the insulin molecule (crystalline insulin), the usual insulin effect is seen on O_2 consumption. Krebs & Eggleston found no effect of zinc insulin on respiration with pigeon mince.

It will be noted from Table IV that animals were used in various nutritional states—post-absorptive, fasted for several days, phlorhizinized-fasted and completely depancreatized—in order to study the *in vitro* effect under all possible conditions of insulin deprivation and of lowered carbohydrate utilization. The phlorhizinized-fasted and completely depancreatized dogs show *in vivo* a marked response to the injection of insulin, and one would expect to find this hormone as a limiting factor in tissues excised from these animals.

The failure to obtain insulin effects with other than pigeon breast muscle might have been due to experimental conditions not being optimal. Therefore,

several modifications were made in an effort to obtain more favourable conditions. Since the previous experiments were done at pH 6·8, a trial was performed with cat skeletal muscle at pH 7·2 and 7·8, by varying the phosphate buffer only. Table VII shows that at pH 7·2 respiration was well maintained for a period of 3·5 hr. without any insulin effect. At pH 7·8 respiration fell off more rapidly, and stopped completely by 2·5 hr. During this period there was no difference between the O_2 consumption in the presence of insulin (5 mg./100 ml.) and that in the controls.

Table VII. *Effect of pH on metabolism of minced cat skeletal muscle*

pH	Duration hr.	Phosphate + muscle extract + citrate	
		No insulin	Insulin 5 mg./100 ml.
7·2	3·5	0·90	0·82
7·8	2·5	0·82	0·81

Stare & Baumann [1939] have recently reported that greater insulin effects are obtained with pigeon breast mince if a NaCl-KCl-MgSO₄ solution, a weaker phosphate buffer (*M*/60) and smaller amounts of citrate and muscle juice are used than those employed by Krebs & Eggleston and by ourselves. This type of solution was, therefore, tested with dog skeletal and cardiac muscles (Table VIII) with no evidence of any insulin effect, although the level of respiration of skeletal muscle was higher than with the customary solutions.

Table VIII. *Comparison of respirations (Q_{O_2}) of minced tissues in different solutions*

	Q_{O_2}		Duration of exp. hr.
	No insulin	Insulin 5 mg./100 ml.	
Skeletal muscle: Krebs-Eggleston solution	0·57	0·54	2·0
Stare-Baumann solution	0·62	0·61	
Cardiac muscle: Krebs-Eggleston solution	1·24	1·15	2·2
Stare-Baumann solution	1·20	1·16	

From the data reported, it can be seen that mammalian muscle has a lower metabolic rate, rarely reaching values more than one-third of those of the pigeon mince. In addition, the mammalian experiments were carried out at 37·5°, whereas the pigeon experiments were done at 40°, their normal body temperature. An attempt was made to study both of these factors by carrying out an insulin experiment on cat skeletal muscle at 40°. The insulin did not produce any change (Table IX), but it should be pointed out that the higher temperature did not produce the expected increase in O_2 consumption.

Table IX. *Effect of insulin on respiration (Q_{O_2}) at 40° of minced cat skeletal muscle*

Insulin mg./100 ml.	Q_{O_2} Phosphate + muscle juice + citrate
0	0·90
0·4	0·89
4·0	0·90
20·0	0·88

Another possible limiting factor was the low cytochrome content of mammalian skeletal muscle, as compared with pigeon breast. Junowicz-Kocholaty & Hogness have reported [1939] that the concentration of cytochrome *c* in pigeon breast muscle is unusually high, about 50 $\mu\text{g.}$ per 100 g. That of mammalian cardiac muscle is also quite high, 20 $\mu\text{g.}$ per 100 g., in contrast with the very low concentration of mammalian skeletal muscle (5 to 10 $\mu\text{g.}$ per 100 g.). While it was not very likely that the lack of insulin effects on cardiac muscle could be due to cytochrome lack because of its high concentration in this tissue, cytochrome might have been the limiting factor with mammalian skeletal muscle. Accordingly, the two experiments summarized in Table X were performed to test the effect of two levels of added cytochrome *c* on the respiration of minced cat skeletal muscle. In one series, a slight rise in O_2 consumption was noted in all the solutions containing cytochrome *c*, but in no case was there an insulin effect.

Table X. *Effects of cytochrome c and insulin upon respiration of minced cat skeletal muscle*

Solution	Q_{O_2}
Exp. 1:	
Phosphate + muscle juice + citrate	0.70
Phosphate + muscle juice + citrate + 5 mg./100 ml. Lilly insulin	0.70
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i>	0.76
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i> + 5 mg./100 ml. Lilly insulin	0.73
Phosphate + muscle juice + citrate + 0.1 mg./100 ml. cytochrome <i>c</i> + 0.5 mg./100 ml. Lilly insulin	0.73
Exp. 2:	
Phosphate + muscle juice + citrate	0.79
Phosphate + muscle juice + citrate + 5 mg./100 ml. Lilly insulin	0.72
Phosphate + muscle juice + citrate + 50 mg./100 ml. cytochrome <i>c</i>	0.70
Phosphate + muscle juice + citrate + 50 mg./100 ml. cytochrome <i>c</i> + 5 mg./100 ml. Lilly insulin	0.68

One is tempted to consider another possible explanation for the insulin effect on the basis of a non-specific change. The reason for the sharp falling off of respiration in minced tissue preparations is obscure. In many instances it does not appear to be due to the lack of substrate. Its explanation might lie in the destruction of protein carriers by proteolytic enzymes. Should this prove to be the case, the delay in falling off of respiration achieved by insulin with pigeon breast mince might be due to a temporary interference with proteolysis by the hormone. From the work of Scott on the binding of trypsin by insulin with subsequent inactivation of the hormone, such a mechanism might exist, the insulin binding part of the proteolytic enzyme system of the cell and hence delaying the disintegrating action. For such a mechanism to explain the insulin effect would necessitate a difference of some kind in the proteolytic enzymes of the pigeon muscle as compared with the other tissues studied. It is a concept, however, susceptible of direct study and may throw some light on the changes produced by insulin, as well as on the difference in survival between minced tissue and the tissue slice or strip.

Comparison of the R.Q. of muscle mince and muscle strips or slices

Since the insulin effect was so sharply limited to the pigeon of all the animals studied, it was of interest to examine the qualitative character of the respiratory metabolism of this tissue for a possible explanation of the difference in behaviour. Considerable weight would be lent to the insulin effect in pigeon muscle as a

specific phenomenon if not only the rate of respiration were accelerated by this hormone, but the character of the foodstuffs oxidized changed from a non-carbohydrate to a carbohydrate type. It was also felt that R.Q. studies on minced mammalian tissues would be necessary to rule out the possibility that, even in the absence of respiratory stimulation by insulin, isocaloric shifts in the character of the foodstuffs oxidized might have taken place. R.Q. determinations, using the method of Dickens and Šimer, were made under a variety of experimental conditions.

Table XI. *Respiratory quotients of minced pigeon breast muscle*

	Condition of animal	Solution	R.Q.	Q _{O₂}
1	Fed	Phosphate	0.89	3.82
		Phos. + muscle juice + citrate	0.92	4.96
	Fed	Phosphate	0.95	4.63
		Phos. + muscle juice + citrate	0.91	5.12
3	Fasted 3 days	Phosphate	1.01	3.87
4	"	"	0.98	4.40
5	"	"	0.96	4.03
6	Fasted 3.5 days	"	0.96	2.21
7	Fasted 4 days	"	0.88	2.04
8	Fasted 4.5 days	"	0.93	4.45

It soon became apparent that all minced muscle preparations, even in the absence of added insulin, had high R.Q., indicating almost exclusive oxidation of carbohydrate. Table XI shows the high R.Q. obtained even in the case of fasted animals which would be expected to be burning fat preponderantly. This high oxidation of carbohydrate during a fast is counter to previous experience in this laboratory with the R.Q. of excised muscle strips and cardiac slices from fasted animals. When comparisons of simultaneous R.Q. were made with minced muscle and muscle strips or slices, there was apparent in each instance a significant elevation in the R.Q. of the mince. These results are summarized in Table XII. Muscles both from pigeons and from fasted and depancreatized dogs behaved similarly. The high R.Q. of the fasted pigeon mince is particularly striking in view of the high metabolic rate of these birds, and the anticipated greater depletion of carbohydrate stores. Blood sugar determinations on the pigeon after three- and four-day fasts were surprisingly high: 229, 206, 197, 233 mg./100 ml. in four such animals.

Table XII. *Effect of mincing on muscle metabolism*

Animal	Exp. condition	Tissue		R.Q.	Q _{O₂}
Pigeon	Fasted 3 days	Breast muscle	Slices	0.79	0.81
			Mince	0.96	4.03
Pigeon	Fasted 3.5 days	Breast muscle	Slices	0.87	0.58
			Mince	0.96	2.21
Pigeon	Fasted 4.5 days	Breast muscle	Slices	0.85	0.73
			Mince	0.93	4.45
Dog	Fasted 6 days	Skeletal muscle	Strips	0.76	0.34
			Mince	0.91	1.27
Dog	Fasted 7 days	Skeletal muscle	Strips	0.69	0.33
			Mince	0.91	1.18
Dog	Fasted 10 days	Skeletal muscle	Strips	0.77	0.35
			Mince	0.86	0.76
Dog	Depancreatized 5 days	Skeletal muscle	Strips	0.73	0.43
			Mince	0.85	0.89

Preliminary experiments by Stare & Baumann indicate that, like the duck [Koppanyi *et al.* 1926], the pigeon tolerates pancreatectomy extremely well with no permanent change in the already high blood sugar. Both these birds would thus seem to be much less dependent on insulin than mammals. Some fundamental qualitative difference in the nature of certain metabolic processes might produce the difference in behaviour of the pigeon mince to insulin as compared with tissues from the other animals studied. The evidence, however, suggests that the pigeon is less dependent on insulin than the mammal, and by that token the *in vitro* effect of insulin in the pigeon is difficult to understand.

The high R.Q. of the minced tissue suggests that the effect of insulin is not in varying the nature of the substrate oxidized, but rather in prolonging the O₂ consumption of a tissue already burning carbohydrate almost exclusively. It therefore fails to provide what would be highly desirable evidence of a specific insulin phenomenon.

DISCUSSION

The interesting effect of insulin on the respiration of pigeon breast muscle described by Krebs & Eggleston has been confirmed. The chief divergence in the data obtained by these workers and by ourselves lies in the smaller increase found, which averaged 25% as against 50%. We are inclined to interpret the effect on respiration produced by insulin as not a true "stimulation" of respiration, but rather as a lag in the falling off of respiration which takes place at a time when the O₂ consumption has fallen markedly from its original value. These results were obtained with three different samples of insulin: B.D.H. insulin hydrochloride, an amorphous insulin preparation (Lilly), and crystalline zinc insulin (Lilly). The positive results with the crystalline insulin were unexpected since Krebs & Eggleston were unable to obtain any effect with this substance. We were also unable to find any significant proportionality between the stimulation of respiration and the concentration of insulin above about 1 mg./100 ml.

The insulin effect is, however, absent in skeletal muscle from the chicken, dog, cat and rabbit, and in dog cardiac muscle. Indeed, it is very common to find a depression of O₂ consumption with insulin with these tissues. This depression was not found to be due to the presence of zinc in the insulin used. It is of interest in this connexion that mammalian tissue seems much less sensitive to zinc than pigeon mince. This, as well as the positive results obtained in the pigeon with crystalline zinc insulin, tend to invalidate Krebs & Eggleston's suggestion that the active form of insulin in the body is probably not similar to the zinc compound because of the depressant action of the metal.

Attempts were made to set up conditions which might be more favourable to the manifestation of the insulin effect. These consisted in raising the pH, altering the electrolyte content of the solutions, elevating the experimental temperature from 37.5 to 40° and adding cytochrome *c*. In addition, animals were fasted and depancreatized in order to bring about conditions in which insulin was a definite limiting factor. All of these modifications were unsuccessful. The absence of the insulin effect in the mammalian tissues and in the chicken does not rule out the possibility that it might occur under proper conditions. However, it casts doubt on any generalized significance of the effect as related to the postulated citric acid cycle.

The criterion for establishing specific insulin action is, in this instance, the level of O₂ consumption. Much stronger evidence would be furnished by a situation in which insulin actually changed the nature of the foodstuffs burned from a non-carbohydrate to a carbohydrate type. It will be recalled that in the

whole animal the effects of insulin are not shown by an increased metabolic rate, but rather by a change in foodstuff oxidized. Examination of the R.Q. obtained by mincing pigeon breast, as well as mammalian skeletal muscle, brings out the interesting fact that this treatment serves to elevate the R.Q. immediately to levels approaching exclusive carbohydrate oxidation. This change took place in all tissues studied, even in the fasted and depancreatized animal where the results were not to be expected from the usual effect of these procedures on the character of the foodstuffs oxidized. The additional effect of insulin on the respiration of minced pigeon tissue would appear to be merely a continuation of the carbohydrate type of oxidation which existed in the non-insulin control.

A comparison of the R.Q. of muscle strips or slices with that of the mince is of considerable interest for workers employing any procedure such as mincing or homogenization. Obviously, by the disruption of the cell structure, more than a quantitative change in the metabolic processes is brought about. The R.Q. of the excised strip or slice parallels that obtained with the whole animal, whereas the R.Q. of the mince is strikingly elevated. Disruption of cell structure may result in the liberation of highly reactive carbohydrate systems no longer under control by the intact cellular organization. This fact should be borne in mind when interpreting data derived from tissue mince and extending them by analogy to the living cell.

A possible explanation for the limitation of this phenomenon to pigeon breast muscle may reside in some peculiar metabolic characteristic of the pigeon. Some evidence for this has been offered in the unusual reaction to fasting as evidenced by the high blood sugar and by the apparent resistance of the pigeon to pancreatectomy, as reported by Stare & Baumann. This difference in behaviour as against that of the mammals would have a parallel in the resistance of the duck to pancreatectomy. It is surprising to find a specific insulin effect only in animals so little dependent upon its action, whereas animals for which this hormone is essential should fail to be influenced.

SUMMARY

1. The insulin effect described by Krebs & Eggleston, consisting of the better maintenance of O_2 consumption in minced pigeon breast muscle, is verified.
2. No comparable effect of insulin is manifested on the respiration of minced chicken breast muscle, minced skeletal muscle of the cat, dog and rabbit, or minced dog cardiac muscle.
3. Therefore, it is doubtful if the insulin phenomenon in pigeon breast muscle exemplifies the general mechanism of insulin action on carbohydrate oxidation.
4. The result of disruption of the intercellular organization of muscle by mincing is to stimulate the oxidation of carbohydrate, even in the absence of added insulin.

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CCXXIV. MAINTENANCE NUTRITION IN THE PIGEON. FURTHER EVIDENCE FOR THE PRESENCE OF DIETARY ESSENTIALS IN YEAST AND LIVER AND THEIR RELATION TO VITAMIN B₆

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EVIDENCE in support of the view that at least four components of the vitamin B complex are essential in the normal nutrition of the pigeon has already been reported briefly by us [Carter & O'Brien, 1937, 1, 2]. By treatment of a yeast concentrate with fuller's earth we have obtained an adsorbed fraction (yeast fuller's earth eluate fraction) which exhibits a supplementary relationship in the nutrition of the pigeon with the material remaining in the filtrate after this procedure. Administration of these fractions together to birds already receiving supplements of aneurin and riboflavin resulted in restoration to maximum weight. A similar relationship has been found to exist between our yeast eluate fraction and the filtrate obtained after treatment of a liver extract with fuller's earth. We think it desirable to present in greater detail the evidence for these findings. By the use of methods which differ somewhat from our own, Edgar *et al.* [1938] have obtained fuller's earth eluate fractions of liver and yeast which supplement the activities present in the corresponding fuller's earth filtrates in relation to rat nutrition. With regard to vitamin B₆ they make the important statement "Since the crystalline vitamin completely replaces our eluate fractions in the diet of the rat, it seems probable that vitamin B₆ is the only essential nutrient for the rat present in our yeast and liver eluates. The possibility, however, still exists that other essential nutrients may be common contaminants of our eluate fraction. . . ." Our own preliminary experience of the contribution of vitamin B₆ to the activity of our eluate fractions in regard to the pigeon is reported in the present paper.

Methods

Birds were maintained singly in cages under conditions which have been previously described. They received a basal purified diet [Carter & O'Brien, 1937, 1] and, in addition to other supplements, cod liver oil at regular intervals. Crystalline aneurin, a synthetic i.g. product, has been used in all experiments. Riboflavin was prepared from ox liver using frankonite and lead sulphide as adsorbents. The fuller's earth eluate and filtrate fractions were prepared as follows.

A crude extract of yeast, partially purified by preliminary treatment with lead acetate followed by baryta, was used as starting material. This extract, which corresponded to 100 kg. yeast, was diluted to 100 l. with water, acidified to pH 1 with H₂SO₄ and treated with 400 g. fuller's earth. After stirring for several hours the extract was filtered on Büchner funnels, the pH of the filtrate

was adjusted to 1.0 and three further treatments with fuller's earth were carried through. The fuller's earths were washed separately and then extracted 4-5 times with a mixture of pyridine, methyl alcohol and water (1 : 1 : 4). The combined eluates were concentrated *in vacuo* to remove pyridine and methyl alcohol, the residue was taken up in a small volume of water and 4 vol. of ethyl alcohol were added. The clear filtrate obtained after the removal of a large gummy precipitate was concentrated to remove alcohol, and, after adjusting the pH to 1, was thoroughly shaken four times with 3 vol. amyl alcohol to remove traces of filtrate factor. The aqueous fraction was then separated, traces of amyl alcohol were removed and the volume was adjusted so that 1 ml. corresponded to 1 kg. yeast. This fraction will be described as the yeast fuller's earth eluate fraction. Attempts were made to obtain a preparation of the filtrate factor from the filtrate of the yeast extract after its treatment with fuller's earth by adsorption on charcoal at pH 1 and 7; they were unsuccessful. An active preparation of the yeast filtrate factor was obtained by submitting Peter's eluate from yeast to successive fuller's earth treatments. The filtrate was concentrated and alcohol added to 50 % concentration in order to remove excess of inorganic constituents. A similar fractionation has been carried out on alcoholic extracts of ox liver after the preliminary removal of ether-soluble material and phosphatides. It has been found possible to remove all but the smallest traces of riboflavin by frankonite treatment at pH 7. Thereafter successive treatments with fuller's earth at pH 1 as described above were employed. In this way a liver fuller's earth eluate fraction and a corresponding filtrate (vitamin B₂) were obtained.

The activity of the above fractions has been tested on pigeons previously depleted on the basal diet alone to a weight level of about 70 % of the maximum and which have thereafter made a partial recovery to a steady submaximal plateau following daily additions of 40 μ g. aneurin and 80 μ g. riboflavin. The duration of the initial period is of cardinal importance in the demonstration of the need for eluate and filtrate factors by the pigeon, since these factors are stored much more tenaciously than is aneurin. After short periods of depletion supplements of aneurin alone may ensure complete weight recovery in a considerable proportion of cases. Only birds whose recovery with aneurin and riboflavin was incomplete were employed in testing the activities of the eluate and filtrate fractions.

RESULTS

Effects of fuller's earth eluates of yeast and liver

Table I shows the response obtained with daily supplements of yeast fuller's earth eluate. The selected group of results is typical of those which have been

Table I

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
17	436	377	436	59	4	15
7	422	373	424	51	6	8.5
780	428	374	445	71	7	10
568	420	382	430	48	6	8
583	320	271	329	58	9	6.4
28	382	330	343	13	5	2.6
533	399	290	304	14	8	1.7
846	436	361	378	17	6	2.8
264	412	278	321	43	13	3.3
700	452	334	416	82	10	8.2 Subsequent decline on continuing eluate

observed in 25 birds. In about 25 % of these the ensuing weight rise is maximal while in the remainder a definite but incomplete response is seen. The results are comparable with those reported for vitamin B₅ concentrates [Carter & O'Brien, 1937, 2]. We have been at pains to exclude possible contamination with traces of factors which are present in the filtrate fraction. Thorough washing of the fuller's earth after adsorption and exhaustive extraction with amyl alcohol of the eluate presumably ensures this, but we have had in mind the possibility that rice starch in the basal diet may carry traces of the filtrate factors. We are at present engaged in further work to test this point. Table II shows similar effects for the liver fuller's earth eluate fraction.

Table II

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
782	380	305	365	60	8	7.5
8	400	314	384	70	32	2.2
568	422	374	411	37	14	2.6
814	500	314	378	64	12	5.3
263	380	247	339	92	7	13
20	500	409	442	33	10	3.3
536	432	326	385	59	9	6.5
3	462	311	356	45	7	6.4
777	500	422	456	34	8	4.2
468	500	368	417	49	17	2.9

With this preparation also restoration to maximum weight occurs in a certain proportion of cases (17 %) and in the remainder the response is incomplete.

Effects of fuller's earth filtrates of yeast and liver

The effects of administration of fuller's earth filtrate from yeast and liver are summarized in Table III.

Table III

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
Effect of yeast filtrate						
531	491	430	454	24	11	2.1
605	500	383	389	6	5	1.2
661	420	320	345	25	8	3.1
103	380	270	271	1	15	Nil
899	412	357	366	9	5	1.8
Effect of liver filtrate						
44	500	386	408	22	7	3.1
14	458	405	445	40	10	4
4	460	374	384	10	15	0.7
899	412	357	366	9	5	1.8
881	500	357	358	1	7	Nil

In the absence of the eluate fraction our earlier preparations of yeast filtrate sometimes restored maximum weight. This may have been due to incomplete removal of factors adsorbable on fuller's earth, since later preparations of yeast or liver filtrates which had been submitted to at least four successive treatments with fuller's earth gave an almost negligible response.

Effects of fuller's earth eluates and filtrates of liver and yeast

The slight response to the filtrate fractions administered alone is not to be taken as evidence of their inactivity for the pigeon. In cases where birds had failed to regain maximum weight after receiving the yeast eluate fraction a further supplement of either yeast or liver filtrate resulted in complete restoration of weight. Some typical results are summarized in Table IV.

Table IV

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
Effect of liver filtrate after yeast eluate						
28	382	343	377	34	14	2.3
595	364	264	360	96	15	6.4
535	393	290	366	76	14	5.3
263	400	331	383	52	8	6.5
416	452	321	415	94	26	3.6
Effect of yeast filtrate after yeast eluate						
21	420	383	404	21	9	2.3
899	412	366	390	24	16	1.5
780	428	391	410	19	13	1.4
Effect of liver filtrate after vitamin B ₆						
1	415	339	386	45	10	4.5
12	460	347	389	42	17	2.5
17	436	399	432	34	6	5.6

It is noteworthy that the response to liver filtrate in three birds which had previously received 40 μ g. crystalline vitamin B₆ daily is comparable with that seen when yeast eluate had preceded administration of the filtrate. The average gain in weight (40 g.) is about three times greater than the average gain in weight (14.5 g.) of a group of six birds which received the same preparation of liver filtrate alone.

A similar restoration to maximum weight is observed in the great majority of birds which received yeast eluate after a preliminary response to yeast or liver filtrate. This is illustrated in Table V.

Table V

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
Effect of yeast eluate after liver filtrate						
44	500	405	500	95	12	7.9
881	500	358	491	133	18	7.4
446	360	295	359	64	9	7.1
899	412	321	407	86	8	10.7
12	460	389	453	64	10	6.5
Effect of yeast eluate after yeast filtrate						
447	334	309	355	46	3	15.3
641	460	428	456	28	7	4
531	491	441	490	49	5	9.8
624	412	369	406	37	12	—
605	500	389	468	79	20	3.9

Effect of vitamin B₆

It is interesting to compare the response to crystalline vitamin B₆, now to be described, with the results already recorded for the fuller's earth eluate. The influence of vitamin B₆ in daily doses of 40 µg. as a supplement to aneurin and riboflavin is shown in Table VI. It has only been possible to observe the behaviour of three birds owing to lack of material, and any conclusion must necessarily be provisional. Nevertheless, both from these results and from those in which vitamin B₆ followed administration of liver filtrate, we have gained the impression that the response to the crystalline product is less marked than that to the yeast fuller's earth eluate fraction. Table VI also illustrates the response to vitamin B₆ following administration of liver filtrate. The result leaves little room for doubt that vitamin B₆ is an essential nutrient for the pigeon. In this series of seven birds tested, all showed a definite response, though in only two can it be said that full weight restoration was obtained. It is possible that nicotinic acid may have been a limiting factor, since, in agreement with Harris [1939], we have found that increases in weight follow administration of large doses of this substance. Our findings in this connexion will be reported later.

Table VI

Bird	Max. wt. g.	Init. wt. g.	Final wt. g.	Gain in wt. g.	Days	Gain in wt. per day g.
Effect of vitamin B ₆						
1	415	339	339	0	10	Nil
12	460	312	347	35	11	3.1
17	436	369	399	30	12	2.5
Effect of vitamin B ₆ following liver filtrate						
7	422	344	420	76	11	7
122	370	316	367	51	7	7.3
13	390	318	350	32	11	2.9
4	460	334	384	50	11	4.5
11	490	440	455	15	9	1.6
173	440	411	422	11	4	2.7
180	427	365	379	14	4	3.5
Effect of vitamin B ₆ following nicotinic acid and liver filtrate						
5	345	271	343	72	7	10.3
523	459	389	455	66	5	13.2
7	500	471	493	22	3	7.3
3	376	358	366	8	4	2.0

In four birds which received vitamin B₆ following 5 mg. nicotinic acid and liver filtrate daily there was a marked response and almost complete restoration of weight in each case.

DISCUSSION

The data reported in this paper support the conclusion which we drew from results previously published [Carter & O'Brien, 1937, 2] that the pigeon requires at least two aqueous soluble factors in addition to aneurin and riboflavin. These additional factors are present in a fuller's earth eluate fraction (vitamin B₆ concentrate) and a fuller's earth filtrate fraction (vitamin B₃ concentrate) respectively. We have shown in unpublished experiments that our fuller's earth eluate fraction contains vitamin B₆, as indicated by its curative action upon rat dermatitis, whereas the filtrate fraction has no such effect. The supplementary action of vitamin B₆ in pigeon nutrition upon the effect of the filtrate factor compared with a similar action of the yeast eluate fraction indicates that vitamin B₆ contributes to the activity of vitamin B₆ concentrates. That it does not

account for the whole activity of the eluate fraction is suggested by our present evidence. This evidence indicates that the effect of vitamin B₆ is not so great as that of the yeast eluate fraction. Two possible reasons may account for this: the administration of inadequate doses of vitamin B₆, or the presence of one or more other active substances in the yeast eluate concentrate. The absence of appreciable further improvement in weight following increase in the dose of vitamin B₆ to 80 μ g. seems to exclude inadequate intake of this substance. Alternatively, the presence of an additional factor in the eluate fraction must be considered. In this connexion it may be noted that vitamin B₆ is more effective in restoring maximum weight when nicotinic acid is administered than when it is not. While more evidence would be required to prove that it is this substance which, together with vitamin B₆, accounts for the full activity of the yeast eluate concentrate, attention may be drawn to the observation of Macrae & Edgar [1937] that nicotinic acid is present in eluate fractions similar to our own, while Harris [1939] has recently reported that nicotinic acid is required by the pigeon.

The weight-restorative action of our most recent preparations of the liver filtrate factor when given without the eluate fraction is negligible in comparison with that of the earlier preparations which had received less exhaustive treatment with fuller's earth. It is possible that the difference is due to the removal of nicotinic acid by this procedure. Such a liver filtrate fraction, however, shows marked activity when supplemented by the eluate fraction or by vitamin B₆. Further experiments are now in progress to substantiate these points.

SUMMARY

1. Evidence is presented to show that the pigeon requires, in addition to aneurin and riboflavin, a fuller's earth eluate factor and a fuller's earth filtrate factor. Both of these are present in yeast and liver. Methods are described for the preparation of concentrates of these factors.
2. The fuller's earth eluate fraction from yeast has a marked weight-restorative action, and when supplemented by the filtrate fraction weight recovery is maximal.
3. Crystalline vitamin B₆ in daily doses of 40 μ g. also exhibits a supplementary relationship with the filtrate fraction. The activity of vitamin B₆ is less than that of the yeast eluate fraction. The possible reasons for this discrepancy are discussed.
4. The fuller's earth filtrate fraction of yeast or liver has little weight-restorative action when supplemented by aneurin and riboflavin, but its activity can be demonstrated when the yeast eluate fraction or vitamin B₆ is given in addition.

We wish to record our grateful thanks to Dr T. F. Macrae, Dr Lepkovsky and Merck and Co., Inc., U.S.A. for generous gifts of crystalline vitamin B₆, and to the Agricultural Research Council for a grant towards the expenses of this research. We thank Prof. R. A. Peters for his continued interest and advice.

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CCXXV. THE ESTIMATION OF VITAMIN B₁ IN CEREBRO-SPINAL FLUID

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(Received 27 September 1939)

THE most sensitive method for the estimation of vitamin B₁ is the one described by Schopfer; this consists of growing a fungus, *Phycomyces blakesleeanus*, upon a synthetic medium to which the substance to be assayed is added. The method has been applied to blood by Meiklejohn [1937], but the test is somewhat invalidated by the presence in blood of other substances, particularly protein, that affect the growth of the fungus [Sinclair, 1938]. For clinical purposes the estimation of the vitamin in plasma or cerebro-spinal fluid might be very useful; the former, however, cannot be done with sufficient accuracy because the amount of vitamin B₁ in plasma is very small (about 1 μ g./100 ml.) and is greatly affected by slight haemolysis of the blood [Goodhart & Sinclair, 1939]. Therefore estimations upon cerebro-spinal fluid were attempted, and the results on 110 cases have already been published [Sinclair, 1939, 1].

Karrer [1937] had previously reported that he found no vitamin B₁ in normal c.s.f.; but he used the thiochrome method which was not sensitive enough to detect it. Kasahara *et al.* [1938], who tested the c.s.f. of rabbits, to which 1 to 4 mg. vitamin B₁ had been given intravenously, by the crude method of injecting 1 ml. of the fluid into polyneuritic pigeons, found none in the c.s.f. of normal rabbits but found it in the c.s.f. of meningitic rabbits. Villela [1939] has reported results obtained by adsorbing the vitamin from 10 to 15 ml. of c.s.f. on frankonite and then testing the adsorbate either by the thiochrome method or by Schopfer's method; the latter method gave higher values than the former. No details of the method have yet appeared and it seems doubtful if the fungus would elute all the vitamin from the frankonite. The values found in 30 cases of mental disease averaged 2.5 μ g./100 ml.; the range is not given. Villela states that "In all cases in which the chemical test was negative, it was possible to detect the vitamin with the *Phycomyces* test." Later this is contradicted by the statement that in some cases "no trace of aneurin could be found by either test".

EXPERIMENTAL

The method has been similar to that already described for blood [Sinclair, 1938]. Amounts of c.s.f. between 1 and 4 ml. are added to flasks containing the medium (with 0.4% asparagine), the flasks are sterilized by tyndallization and inoculated with a suspension of *Phycomyces blakesleeanus*. The weight of the fungus produced after 10 days is proportional to the amount of vitamin present.

The only substances that are known to promote growth of the fungus are vitamin B₁, its phosphate esters, its degradation products and certain substances very closely related to these. That the slight growth obtained with c.s.f. is due to vitamin B₁ or its phosphate esters is indicated by the observation that no growth is obtained if the vitamin is converted into thiochrome by alkaline ferricyanide (Table I).

Table I. *Effects of alkali and ferricyanide upon the growth factor in c.s.f.*

C.s.f. ml.	Vitamin B ₁ μg.	K ₃ Fe(CN) ₆ (1%) drops	NaOH (5N) drops	Wt. of fungus mg.	Vitamin equivalent μg.
0	—	—	—	0	0
0	0.1	—	—	23.5	0.10
0	2.5	—	—	106.5	Excess
1	—	—	—	3.2	0.003
1	0.1	—	—	24.2	0.105
1	2.5	—	—	110.2	Excess
2	—	—	—	6.6	0.007
2	0.1	—	—	25.8	0.11
2	2.5	—	—	116.5	Excess
2	—	—	1	6.2	0.006
2	0.1	—	1	22.2	0.095
2	0.1*	—	1	23.1	0.10
2	—	2	1	0	0
2	0.1	2	1	0	0
2	0.1*	2	1	23.9	0.105

* Vitamin added after alkali.

In experiments such as the one summarized in Table I, c.s.f. and then 2 drops of 1% potassium ferricyanide followed by 1 drop of 5N NaOH were added to the flasks, which were then placed in boiling water for 1 min. After cooling, the pH was adjusted to 6.5 with HCl; the medium was then added and the flasks sterilized by tyndallization. In control flasks vitamin was added either before or after the ferricyanide to show that the treatment was sufficient to destroy the growth-promoting properties of the vitamin without the reagents themselves altering the growth of the fungus in presence of vitamin subsequently added. It will be seen from Table I that heating with alkali does not abolish the growth produced by c.s.f., no doubt because this treatment breaks vitamin B₁ into its constituent parts which are together active [Sinclair, 1937]. Heating with alkaline ferricyanide, however, abolishes the growth produced by c.s.f.; this treatment oxidizes the vitamin to thiochrome, which does not act as a growth factor for the fungus [Meiklejohn, 1937].

I have already shown that blood contains substances other than vitamin B₁ that affect the growth of the fungus [Sinclair, 1938]. But since protein is probably the most important of these, c.s.f. might be expected to give more satisfactory results than blood. Experiments show that this is the case. Unlike blood, c.s.f. is unable to promote growth of the fungus in absence of added medium; and if in presence of c.s.f. the asparagine is omitted from the medium so that it contains no source of nitrogen, lower weights of fungus are obtained and there is no effect of added vitamin B₁. Whereas relatively higher values for vitamin B₁ in blood are obtained when hydrolysed casein (0.8%) is substituted for asparagine in the medium, identical values are obtained with c.s.f. Unlike blood, samples of c.s.f. up to 4 ml. give the same values per unit volume (Table II), and the growth obtained by adding 0.1 μg. vitamin B₁ to samples of c.s.f. is the same as (or very slightly more than) would be expected from the result obtained without added vitamin (see Table I).

However, it is easy to show that there is usually an adjuvant factor in c.s.f., although very much less than in blood. If excess vitamin B₁ (2.5 μg.) be added to samples of 1–4 ml. c.s.f., the growths obtained are usually larger than those in the control flasks without added c.s.f.; results with 1 and 2 ml. c.s.f. are summarized in Table III and compared with similar results for blood [Sinclair, 1938].

Table II. *Effect of size of sample of c.s.f. upon estimated content of vitamin B₁ ($\mu\text{g.}/100\text{ ml.}$)*

Sample	ml. c.s.f.			
	1	2	3	4
	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
1	0.2	0.2	0.2	—
2	0.5	0.7	0.7	—
3	0.5	0.5	0.7	—
4	0.6	0.7	0.7	0.8
5	0.7	0.7	0.7	—
6	1.3	1.5	1.8	—
7	1.5	1.6	1.7	—
8	2.0	2.5	2.3	2.4
9	3.0	3.5	3.3	—
10	5.0	4.0	3.0	—
11	5.5	5.5	4.3	—
Mean	1.89	1.95	1.76	

Table III. *Effect of c.s.f. upon the growth of Phycomyces in presence of excess vitamin B₁*

Vol. of c.s.f. ml.	No. of observations	Increase in presence of c.s.f. (%)	
		Range	Mean
1	110	0-78	22
2	105	0-116	37
Blood 1 ml.	50	14-160	78

That part of this adjuvant effect of added c.s.f. is due to the fluid buffering the medium is indicated by Table IV. In this table a comparison is made of the pH of the medium after the fungus has stopped growing in presence of c.s.f. or in presence of approximately the same amount of vitamin. It will be seen that the change in pH from the original 6.5 is less in the flasks containing c.s.f. than in the controls.

Table IV. *Effect of c.s.f. upon the final pH of cultures of Phycomyces*

C.s.f. ml.	Added vitamin B ₁ $\mu\text{g.}$	Approx. conc. of vitamin present $\mu\text{g.}$	Final pH	Added vitamin B ₁ $\mu\text{g.}$ (no c.s.f.)	
					Final pH
1	—	0.035	4.8	0.025	4.0
1	0.1	0.15	3.8	0.15	3.3
2	—	0.07	4.6	0.10	3.5
2	0.1	0.165	3.8	0.20	3.3
1	2.5	Excess	4.6	2.5	3.7
2	2.5	Excess	4.8		

Because of this adjuvant effect, the values for vitamin B₁ that are given by the test are higher than the true values. But it will be shown in a paper now in preparation that similar apparent values for blood can be corrected to give approximately true values by multiplying the weights of fungus produced in the presence of, say, 1 ml. blood by the ratio of the weight obtained with excess vitamin to the weight obtained with 1 ml. blood in presence of excess vitamin; the corrected weight thus obtained is then used to obtain the true value from the growth-vitamin curve. For instance, taking the figures given in Table I, we obtain the corrected weight for 1 ml. c.s.f. by multiplying the observed

weight (3.2 mg.) by $\frac{106.5}{110.2}$, and the corrected weight for 2 ml. by multiplying 6.6 mg. by $\frac{106.5}{116.5}$. The corrected weights are 3.1 and 6.0 mg. respectively, equivalent to 0.003 and 0.006 μ g. vitamin B₁. The weights obtained in the presence of small amounts of added vitamin (0.1 μ g. in Table I) can be corrected in the same way. It is believed that this method gives approximately true values for the estimation of vitamin B₁ in 1–4 ml. samples of c.s.f.

The method described above estimates both free vitamin B₁ and cocarboxylase; normal c.s.f. contains none of the latter [Goodhart & Sinclair, 1939]. I have previously shown that estimations on blood are much affected by the temperature to which the blood is heated; very low values are obtained with unheated blood because most of the vitamin is combined with protein. This is not true of c.s.f. If sterile c.s.f. be added to flasks that have previously been filled with medium and sterilized and then the flasks be inoculated without further heating, the same values are obtained as when the flasks are tyndallized before inoculation. This shows that the vitamin in c.s.f., unlike that in blood, is uncombined. This conclusion is confirmed by the fact that all the vitamin in c.s.f. is diffusible [Sinclair, 1939, 2].

RESULTS

The results already quoted, with the exception of those in Tables II and III which include all relevant estimations, have been obtained with samples of human c.s.f. that have shown no marked pathological features. The method has been applied to 272 different samples of c.s.f. obtained from hospital patients. Excluding all the samples that either showed marked pathological features (increased protein or cells, or non-sterility) or came from patients who might be deficient in vitamin B₁, the values (in μ g./100 ml.) were as follows:

	No. of observations	Range	Mean ($\pm \sigma$)
Apparent	177	0.0–2.0	0.7 \pm 0.5
True	150	0.0–1.3	0.5 \pm 0.4

Taking the limits of $\pm 2\sigma$, it may be said that the amount of vitamin B₁ in the c.s.f. of “normal” individuals is 0.0–1.3 μ g./100 ml., mean 0.5 μ g. It happens that this statistical variation is the exact range within which all the 150 true values have fallen.

The corresponding figures for the sterile pathological samples were as follows:

	No. of observations	Range	Mean ($\pm \sigma$)
Apparent	95	0.1–12.0	3.5 \pm 2.0
True	83	0.1– 6.5	2.2 \pm 1.3

It will be seen that these values tend to be much higher, and it is instructive to correlate the values with the different abnormalities in the samples. A mere increase in protein in the fluid does not usually cause abnormally high true values for vitamin B₁: for instance, samples of c.s.f. withdrawn on different days from a patient with an intracerebral tumour contained 0.2, 0.8, 0.2 and 0.1 μ g. vitamin B₁ per 100 ml., and the corresponding protein values were 320, 360, 300 and 340 mg./100 ml. (the second sample contained 2240 red blood corpuscles per μ l.); it is interesting to mention here that ascitic fluid containing 1600 mg. protein per 100 ml., from a patient with mitral stenosis, contained no vitamin B₁. But increase in the cells is accompanied by abnormally high values for the vitamin. Therefore high values are found in cases of meningitis, as shown in Table V, which includes all the sterile samples that were obtained from such cases.

Table V. *Variation of vitamin B₁ with cell count in c.s.f. from cases of meningitis*

Case no.	Type	White cells per μ l.	Differential count (%)		Red cells per μ l.	Vitamin B ₁ μ g./100 ml.
			Neutrophils	Lymphocytes		
1	Meningococcal	2850	88	12	0	4.6
2	Meningococcal	548	67	28	0	3.5
		287	100	—	0	3.5
		278	59	30	0	3.4
		61	31	68	0	1.5
3	Tuberculous	154	—	100	127	3.0
4	Meningococcal	130	46	54	1606	4.0
		127	74	26	86	3.0
5	Pneumococcal	38	72	38	0	0.0
		27	40	95	0	0.4
6	Tuberculous	17	—	100	1200	1.5
		9	—	100	580	0.1
		6	—	100	780	0.6
		3	—	100	260	0.5
		1	—	100	180	0.7
7	Pneumococcal	6	—	100	0	0.5
8	Tuberculous	1	—	100	0	0.5

A statistical analysis of the figures in Table V shows that there is a positive correlation between the white cell count and the vitamin content: the correlation coefficient is 0.59 ± 0.16 . An analysis by the method of partial correlation fails to demonstrate any significant effect of the red cell count on the vitamin content in these particular figures: the correlation coefficient is 0.06. The regression of amount of vitamin B₁ on white cell count calculated from the figures in Table V indicates that a single white cell contains 1.4×10^{-8} μ g. vitamin B₁. These results agree well with our earlier conclusions. Mammalian leucocytes were shown to contain much more cocarboxylase than erythrocytes: as a very rough approximation it was stated that one leucocyte, in a sample of ox blood, contained as much cocarboxylase as 1600 erythrocytes [Goodhart & Sinclair, 1939]. In estimations of the true vitamin B₁ in the blood of 45 healthy adults, I have obtained a mean ($\pm \sigma$) of 7.4 ± 1.4 μ g./100 ml.; and plasma contains about 1 μ g./100 ml. Therefore, assuming a blood count of 5×10^6 r.b.c. and 7×10^3 w.b.c. per μ l. and that plasma represents 50% by volume of whole blood, we have: $7.4 = 0.5 + \left(\frac{5 \times 10^6}{1600} + 7 \times 10^3 \right) \times x \times 10^5$, where x is the mean amount of vitamin B₁ (in μ g.) in one leucocyte. From this equation $x = 0.7 \times 10^{-8}$, which is of the same order as 1.4×10^{-8} deduced above. In fact a higher figure should be obtained with the figures for meningitic c.s.f. than with figures for normal blood, since the former fluid is likely to contain disintegrated leucocytes. From these results it appears very probable that the increased values for vitamin B₁ obtained with pathological samples of cerebro-spinal fluid are mainly due to the increased cell count, particularly the white cell count. These results obtained with c.s.f. therefore agree very well both with the estimations of cocarboxylase in blood made by a chemical method and with the estimations of total vitamin B₁ made by a biological method.

DISCUSSION

It is known that vitamin B₁ acts in the body as a catalyst, necessary for the degradation of pyruvate, only after it has been converted into its diphosphate ester, cocarboxylase. The vitamin circulates in plasma in the free unphosphory-

lated form; in this form it diffuses readily and phosphorylation of it with formation of cocarboxylase occurs inside the cells. It has been shown that the vitamin B₁ in normal c.s.f. is free and unphosphorylated; its concentration, 0.0–1.3 $\mu\text{g.}/100\text{ ml.}$, is of the same order as the amount of free unphosphorylated vitamin in human plasma or blood [Sinclair, 1939, 2]. The cells of human blood contain about seven times as much vitamin as plasma, most of the vitamin in them being phosphorylated. Therefore it is not surprising that in pathological samples of c.s.f., with an increased number of cells, the amount of vitamin tends to be increased (as discussed above) and cocarboxylase may be found [Goodhart & Sinclair, 1939].

Unfortunately, estimations of the vitamin in c.s.f. seem to be useless for clinical purposes; the amount is extremely small, and zero values have been obtained in 22 patients who were almost certainly not deficient in the vitamin (such as patients with intracerebral tumours on normal diets and with no vomiting). It may be mentioned, however, that positive values have been found in untreated cases of diabetic neuritis, subacute combined degeneration of the cord and sciatica. The highest values in sterile c.s.f. have been obtained in cases of meningitis, cerebral abscess, intracranial tumour and hydrocephalus.

SUMMARY

1. A method is described, based upon the work of Schopfer, of estimating vitamin B₁ in 1–4 ml. samples of cerebro-spinal fluid. The method gives approximately true values.

2. In normal c.s.f. the vitamin is in the free unphosphorylated form.

3. The method has been applied to 272 different samples of c.s.f. obtained from hospital patients. The normal range is 0.0–1.3 $\mu\text{g.}/100\text{ ml.}$, with a mean ($\pm \sigma$) of 0.5 ± 0.4 .

4. Pathological samples of fluid tend to give higher values; the values show a positive correlation with the white cell count.

5. These results are discussed in relation to other work on the metabolism of vitamin B₁.

6. The method seems to be useless for clinical purposes.

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CCXXVI. THE FATE OF STRONTIUM AFTER INTRAVENOUS ADMINISTRATION TO NORMAL PERSONS

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THE intestine generally, and the large intestine in particular, has long been thought to excrete Ca, Mg and Fe, and by so doing to play a large part in regulating the amounts of these metals within the body. In the last few years, however, several investigators have tested this theory experimentally, and shaken it to its foundations. It now appears probable that the intestinal excretion of these metals is small and may even be negligible. The intestine certainly has not the regulatory function once assigned to it. The subject has been briefly reviewed by McCance & Widdowson [1939], who have themselves injected Fe, Ca and Mg into normal persons, and failed to obtain any evidence of increased excretion by the intestine.

High concentrations of Sr are not unknown in biological tissues [Fox & Ramage, 1931; McCance & Masters, 1937], but in mammals only traces have been found [Gerlach & Müller, 1935]. Chemically, however, and to some extent physiologically [Haldane, 1925; Gerlach & Müller, 1935], Sr resembles Ca and it was felt therefore that an investigation of its excretion by man might not only be of interest in itself but might also throw light on the way in which Ca is excreted.

The literature shows that Sr has been used medicinally for a very long time. According to Loeser & Konwiser [1929-30] it was introduced into therapeutics by Vulpian in 1885, and in comparatively recent times it has been given both by mouth and vein in the treatment of a variety of diseases [Hesse, 1926; Hummel, 1928; Gassmann, 1938, etc.]. The elimination of Sr seems scarcely to have been studied, although Loeser & Konwiser [1929-30] state without reference that Sr "is eliminated by the intestines. The urine contains only traces of strontium when given subcutaneously". Haldane [1925] showed that after taking 60 g. of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ by mouth—which caused violent diarrhoea—1.46 g. of Sr (7.4 % of the amount taken) were excreted in the urine over the next 24 hr. No further urines were collected.

Experimental arrangement and technique

R. A. M., aged 40, male, and E. M. W., aged 32, female, were themselves the two subjects, and the experiments were timed to avoid E. M. W.'s menstrual periods. To obtain the basal excretion of Sr, if any, we arranged that for 11 preliminary days we should eat only simple foods which could be duplicated during later periods. After 3 days of this regime urine and faeces were collected for a period of 7 days. It was originally intended that during the experimental period the dietary programme should be repeated, that Sr should be injected intravenously each day for 1 week, and that the urine and faeces covering this period should be collected and examined for Sr. No after period was planned because,

when Ca had been given intravenously, the extra Ca excreted during the experimental period was equal to the amount injected. This scheme was carried out until five injections had been given; but then, owing to an accident, which came about in the following way, the subsequent arrangements had to be modified. At the commencement of the experimental period a solution of strontium lactate was made up containing 23.5 mg. Sr/ml. and enough sterilized, as was thought, to last till the end of the experiment. The solution, which appeared opalescent after being autoclaved, was injected slowly into an antecubital vein. 23.5 mg. were given to E. M. W. on the first day of her experiment but from the second day onwards it was decided to double the dose. R. A. M. commenced his experiment 1 day after E. M. W. and received 47 mg. on each day. Hence by the 5th day it was necessary to sterilize more of the original solution, which by this time had itself become slightly opalescent. This further sterilization was carried out without appreciating that the original solution had become heavily infected with bacteria. We gave the newly sterilized solution intravenously to each other about 11 to 11.30 a.m. on the 5th day of R. A. M.'s experimental period and on the 6th day of E. M. W.'s. In just under an hour we began to feel very ill. E. M. W. had a most intense headache, followed by pains in the back and thighs, rigors, vomiting and diarrhoea. R. A. M. had a much milder headache, rigors and severe pains in the back and thighs. Three to four hours after the injection we both had high temperatures which fell slowly throughout the evening and night. Some modification of the diet was unavoidable during this 24 hr., and on the morning following the accident we decided to omit the daily Sr as both of us were still feeling somewhat shaken, and to utilize the remaining days of the time available for this experiment as an after period. As it turned out this rather improved the experiment. Fortunately, the accident did not prevent the complete collection of the excreta.

During the preliminary period of 7 days the urines for the whole week were mixed for analysis. Toluene was used as a preservative. The faeces were collected in one vessel as described by Widdowson & McCance [1937]. In the experimental period the bladder was emptied each day just before the Sr was given intravenously, and again 2 hr. later. These 2 hr. specimens were mixed under toluene and analysed separately from the urine passed throughout the rest of the 24 hr. The faeces were collected as during the preliminary period. Those passed on the day of the accident by E. M. W., and those passed on the following day by R. A. M. were analysed separately but the results have been included with those of the experimental period in Table I A and B. Duplicate portions of 250 ml. of urine were strongly acidified with H_2SO_4 , evaporated in silica crucibles and ashed at about 450° . The faeces were treated with HNO_3 and ashed [Widdowson & McCance, 1937]. All the ashes were weighed and transferred to small, well-stoppered vaccine bottles.

With the co-operation of Mr N. L. Kent the excretion of Sr was followed spectrographically. Such a method has not the accuracy of a good chemical determination, but it has advantages which were considered to outweigh this. Firstly, spectrographic methods are highly specific, and secondly they are exceedingly "micro". Both these attributes are most important in following "trace" elements, and in this study they enabled much smaller quantities of Sr to be injected during the experimental period than would otherwise have been necessary. Thus the general metabolism of Ca was less likely to be upset. The determinations could not be made by Ramage's filter paper method [Sheldon & Ramage, 1931] because the Sr lines did not appear satisfactorily on the plate even when Sr was known to be present in the sample, or had been

added. In passing it may be mentioned that since Sheldon & Ramage employed this method, they may have failed to detect Sr in many of the mammalian tissues examined by them. The determinations were therefore made by the arc method. Brass electrodes were used and a great many exposures were made to get the "unknowns" and the "standards" close to each other both in strength and position. The standard solutions were made by adding known amounts of Sr to the urine of R. A. M.'s preliminary period.

Results

Table I A shows the results of R. A. M.'s experiment. It will be seen that during the preliminary period a little more Sr was found in the urine than in the faeces, and that the weekly excretion amounted to 24 mg. The urinary output for the 2-hourly experimental periods which followed the injection of Sr was 15-20 times higher than it had been during the preliminary period, and an output 10 times as great was maintained throughout the rest of the experimental period. The faecal excretion was barely doubled. A high rate of urinary excretion was maintained throughout the after period, and by the end of the experiment 57 % of the injected Sr had been excreted. 93 % of the "recovered" Sr was eliminated by the kidney.

Table I. *Strontium excretion, R. A. M. (A) and E. M. W. (B)*

	A mg.	B mg.
Preliminary period (7 days):		
Urinary excretion of Sr/hr.	0.084	0.183
Faecal excretion of Sr/hr.	0.058	0.042
Sr excreted in the preliminary period	24	38
Experimental period (R. A. M. 5 days; E. M. W. 6 days):		
Urinary excretion of Sr/hr. for the 2 hr. following Sr injections ...	1.40	0.73
Urinary excretion of Sr/hr. throughout the rest of the 24 hr. ...	0.82	0.69
Faecal excretion of Sr/hr.	0.11	0.099
Sr excreted in the experimental period	118	114
After period (2 days):		
Urinary excretion of Sr/hr.	0.72	0.29
Faecal excretion of Sr/hr.	0.12	0.0
Sr excreted in the after period	40	14
Sr injected	235	258
Sr recovered (Sr excreted in the experimental and after periods minus the amount excreted in the preliminary period) ...	134	85
Sr recovered (as % of the amount injected)	57 %	33 %

Table I B shows E. M. W.'s results and in essentials they resemble those of R. A. M. One or two minor differences should be mentioned. Firstly, the urinary excretion of Sr during the preliminary period is surprisingly high when one considers R. A. M.'s urinary/faecal ratio at this time and the well-known urinary/faecal excretion ratios for Ca. The urine may have been contaminated but there is no reason for this assumption. During the experimental period the urinary output of Sr did not rise so high as did that of R. A. M. and the faecal output did not appear to rise at all till the day of the accident when a relatively large output of Sr accompanied the diarrhoea. The average for the 6 days indicates a small increase. Only 33 % of the injected Sr had been recovered by the end of the experiment, and even supposing the preliminary urine to have been contaminated the amount recovered would probably not have exceeded 40 %; 88.5 % of the "recovered" Sr was found in the urine.

DISCUSSION

These results show that the kidney is the organ primarily responsible for the excretion of Sr once it has been absorbed from the gut. They lend no support whatever to the statement in Loeser & Konwiser's [1929-30] article. There are, however, dozens of similar statements about Ca and Fe in the literature, and they are all expressions of the fact that the old theory had been dominant for so long that it had become accepted without question. It is probable from the slow rate at which Sr is excreted that it escapes to the tissues very soon after injection, where possibly it may displace an equivalent amount of Ca, and whence it may only slowly find its way back into the general circulation to become available for excretion. This slow rate of elimination is important, for injected Ca is excreted much more rapidly [McCance & Widdowson, 1939]—possibly because the tissues are already saturated with this element. It was thought that Ca might be eliminated so rapidly by the kidney that the intestinal excretion never had a chance to come into action, and that therefore injecting Ca was not a fair test of the intestinal path of excretion. The same criticism cannot be made of this study. The fact that there is a small increase of Sr excretion in the faeces is an interesting feature of these experiments, although E. M. W. did not show any increase till the day on which the accident happened. Assuming the increase to be a true one, however, it suggests that injected Sr finds its way into the intestinal juices and possibly replaces an equivalent amount of Ca therein. The results in general terms support the view that the intestinal excretion of Ca or Mg is unlikely to play an important part in regulating the amounts of these metals within the body.

SUMMARY

1. 47 mg. of strontium were injected intravenously every day for 5 days into two normal adults. A smaller preliminary dose was given to one.
2. The strontium was excreted slowly and about 90 % of it by the kidney.

Both of us are truly grateful to Prof. and Mrs Ryle for removing us from the Laboratory on 6 June and putting us to bed in their own house. E. M. W. is indebted to the Medical Research Council for a personal grant.

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CCXXVII. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

IV. FURTHER INVESTIGATION OF *O*- β -GLUCOSIDOTYROSYL DERIVATIVES OF PROTEINS

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In their papers on the preparation and serological properties of the *O*- β -glucosidotyrosyl derivatives of various proteins Clutton *et al.* [1937; 1938] showed that antisera against the gelatin and insulin derivatives precipitated with the corresponding globulin derivative, although they failed to precipitate with the homologous antigens. They also found that antisera against the globulin derivative precipitated both with the homologous antigen and with the gelatin and insulin derivatives. From this they concluded that coupling with glucosidotyrosine converts both gelatin and insulin into full antigens. They also concluded from the similarity in behaviour of globulin derivatives of *O*- β -glucosido-*N*-carbobenzyloxytyrosine and *O*- β -glucosidotyrosine that the hapten function of the carbobenzyloxy group was probably negligible.

In view of the theoretical importance of the points involved it was decided to extend these investigations by the use of other serological techniques, namely complement fixation and bulk precipitation, in addition to the ring precipitation tests and inhibition tests used previously. It was also decided to make a closer study of the carbobenzyloxy group as a possible antigenic determinant, since it seemed surprising that this group should have no influence.

Since an antigen built upon the same (foreign) protein had been used in the earlier experiments for immunization and for testing the antisera, it seemed that the resemblances found originally between the immunological behaviours of *O*- β -glucosido-*N*-carbobenzyloxytyrosyl globulin and *O*- β -glucosidotyrosyl globulin might have been due to the protein moiety. The latter objection was overcome by using the appropriate derivative of rabbit serum proteins as test antigen, thereby avoiding the possibility of the production of antibodies against the protein portion. The preparation of the antigens was otherwise similar to that described in the previous papers, except for the preparation of *N*-carbobenzyloxytyrosyl gelatin, and for slight modifications given below.

EXPERIMENTAL

Preparation of compounds

(1) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl gelatin (from Gold Label gelatin), *O*- β -glucosido-*N*-carbobenzyloxytyrosyl globulin and *O*- β -glucosido-*N*-carbobenzyloxytyrosyl insulin were prepared as described by Clutton *et al.* [1937; 1938].

The glucose contents (measured by the orcinol method) were, on a nitrogen basis: gelatin compound (average of 5 preparations) 4.6%; globulin compound (average of 3 preparations) 12% (the original globulin contained 2.5%); insulin compound (average of 3 preparations) 12%.

(2) *O-β-Glucosido-N-carbobenzyloxytyrosyl rabbit serum protein*. Fresh rabbit serum was dialysed against running tap water for 3 days. 25 ml. (containing 1.05 g. protein on a nitrogen basis) were coupled with the azide from 0.7 g. glucosido-*N*-carbobenzyloxytyrosyl hydrazide, the reaction being kept just alkaline to thymolphthalein. The solution was brought to pH 5; next day the precipitate was collected, dissolved in water with the minimum of alkali and dialysed against running tap water for 30 hr. The product contained, on a glucose : nitrogen basis, 7.4% glucose.

(3) *N-Carbobenzyloxytyrosyl gelatin*. Carbobenzyloxytyrosyl hydrazide was prepared from carbobenzyloxytyrosine ethyl ester and hydrazine hydrate dissolved in alcohol at room temperature. 4.5 g. of the hydrazide were dissolved in 22.5 ml. glacial acetic acid plus 112.5 ml. water, and to the solution were added 13.5 ml. of 5*N* HCl. The mixture was cooled in ice, and 4.5 ml. of 30% NaNO₂ were added very slowly. The azide formed a white precipitate which was immediately extracted with a little ether; the ethereal solution was washed 4 times with water and added slowly, with stirring, to a solution of 10 g. gelatin in 2 l. of 50% aqueous alcohol, kept cool and maintained just alkaline to phenolphthalein by addition of the necessary amount of NaOH. Stirring was continued for an hour after the addition was completed. On acidification with acetic acid the compound flocculated; it was separated on the centrifuge, and washed with 70% and absolute alcohol. Yield, 9.3 g.

Estimation of the tyrosine plus tryptophan content on an alkaline digest by the phenol reagent of Folin & Ciocalteu [1927] showed a tyrosine content of at least 6.5% (since the untreated gelatin contains none). The compound was negligibly soluble at pH < 6, and on boiling with 5*N* HCl gave a definite odour of benzyl chloride.

(4) *Removal of carbobenzyloxy residues*. In the case of the globulin and the rabbit serum protein compounds the technique was similar to that used by Clutton *et al.* [1938], namely reduction with hydrogen in the presence of palladium black. It was found that after 2 hr. shaking with hydrogen at pH 8–9 the compounds were soluble in comparatively acid solution (pH 5.7), and reduction was therefore continued at this pH with the addition of further palladium black. At the end of the reduction the solution just became cloudy on bringing to pH 5.5. After acidification the CO₂ evolved was about 10% greater in amount than was theoretically required for the complete removal of the carbobenzyloxy groups, while in the fourth case it accounted for 85% of these groups. The glucose : nitrogen ratio showed a glucose content of 12.5–14% for the different samples of coupled horse globulin, and 5.3% for the coupled rabbit serum.

Apart from the increased acid solubility and the evolution of CO₂ during the reduction no proof was obtained that the carbobenzyloxy groups had been completely removed. Attempts to assess the degree of removal by measurement of the CO₂ evolved on boiling with 5*N* HCl indicated a considerable (80–100%) removal, but were rendered insufficiently accurate by the fact that the original proteins gave relatively large blanks when treated similarly. In view of the serological results obtained the possibility of a few remaining carbobenzyloxy groups must be borne in mind.

Technique of immunization

Rabbits of 2–3 kg. weight were injected at 2-day intervals. A course of injections comprised three injections of 10 mg., three of 20 mg. and three of 40 mg., the larger doses being given intraperitoneally. Test bleedings were made 8–10 days after the last injection.

The following derivatives were used:

(a) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl gelatin. Originally three animals were used and received two courses. For repeating the experiments a single animal was used, and received two courses, while for the preparation of strongly precipitating antisera (*v. infra*) three animals were used, and received an extended course totalling 600 mg. per rabbit.

(b) *N*-Carbobenzyloxytyrosyl gelatin (two animals). One course.

(c) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl globulin (three animals). One course.

(d) *O*- β -Glucosidotyrosyl globulin (two animals). One course.

(e) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl insulin. The injections were begun on three animals, using the technique described in the previous paper, but only one rabbit survived to the end of the course. One course.

Serological tests

Complement fixation was carried out at 37° in a volume of 1.25 ml. Complement used was 1½–2 M.H.D. fresh guinea-pig serum, and the haemolysin was standard Burroughs Wellcome anti-sheep corpuscle haemolysin used at 2 M.H.D. 1–2 hr. were allowed for fixation before adding the sensitized cells, and readings were taken at 37° for 1 hr., followed by standing in the cold room. Dilutions given are those of the antigen added to the system; the final concentrations of antigen are 1/5 of these values.

Bulk precipitation was carried out at 37° in a volume of 0.5 ml. consisting of equal volumes of serum and antigen dilution. Initial dilutions of antigen are given in the protocols and should be multiplied by 2 to give final dilutions, except where otherwise stated.

Sera were used in dilutions 1 : 2 to 1 : 4 unless stated otherwise, and were heated at 56° for 30 min. before all complement fixation tests.

Dilutions of antigens were made with normal saline, and all antigens were brought to pH 7 with NaOH before testing.

RESULTS

Gelatin derivatives

A. *Ring tests*. The results of Clutton *et al.* [1938] for antisera to G.C.T.¹ gelatin against G.C.T. globulin were confirmed. We also obtained slight indication of a ring at 1/1000 for G.C.T. gelatin antiserum and the homologous antigen.

Antisera to C.T. gelatin gave no ring test against C.T. gelatin, nor against G.C.T. gelatin. Against G.C.T. globulin no ring appeared at a dilution greater than was given by normal serum.

B. *Complement fixation*. Complement fixation was complicated by two factors. One was that G.C.T. gelatin (but not C.T. gelatin) itself caused haemolysis in the presence of complement when used in 1/50 or 1/100 dilution. This was evident when it was added to a known complement-fixing system containing *Brucella* endoantigen and anti-*Brucella* serum. The other was that

¹ In this section of the paper, G. = *O*- β -glucosido, C. = *N*-carbobenzyloxy, T. = tyrosyl.

certain of the normal sera used as controls fixed complement with uncoupled globulin and globulin derivatives, although never to the same dilutions as the antisera. The results were confirmed with a strong serum from a rabbit which was given a double course of immunization, rising to doses of 70 mg. antigen. The results given in Table I indicate that fixation does occur between antisera to G.C.T. gelatin and C.T. gelatin and the corresponding derivatives, but the reactions are weak.

Table I

Antiserum	Antigen	Degree of fixation of complement
G.C.T. gelatin	G.C.T. gelatin	Complete to 1/200
	G.C.T. globulin	Complete to 1/6000, partial to 1/700,000
	C.T. gelatin	Complete to 1/200
C.T. gelatin	C.T. gelatin	Complete to 1/100, partial to 1/900
	G.C.T. gelatin	Complete to 1/200, partial to 1/400
	G.C.T. rabbit serum	Complete to 1/2700
Normal serum	G.C.T. gelatin	None
	C.T. gelatin	None
	G.C.T. globulin	Complete to 1/1000, partial to 1/20,000
	G.C.T. rabbit serum	Complete to 1/300

C. *Bulk precipitation.* In order to try to confirm the existence of a visible reaction between G.C.T. gelatin and its homologous antiserum, a bulk test was set up with the most strongly fixing serum. After 24 hr. a definite opalescence appeared in the tubes containing antigen in 1/50 and 1/100 dilutions, but not in control tubes with normal serum. However, another group of three rabbits, each receiving a total of 600 mg. antigen per rabbit, yielded sera which gave no visible reaction at all under similar conditions.

Insulin derivative

A. *Complement fixation* (serum 1 in 5). Both the anti-G.C.T. insulin serum and normal serum, although the latter to a less degree, fixed complement with the homologous antigen:

Antiserum fixed complement with G.C.T. insulin up to 1/100,000.

Normal serum fixed complement with G.C.T. insulin up to 1/500.

B. *Bulk precipitation.* Anti-G.C.T. insulin serum in five experiments gave a precipitate up to 1/1600 dilution of the antigen. Two fresh normal sera and two old normal sera gave precipitates up to 1/800, 1/400, 1/200 and nil respectively. Thus there was clearly a considerable formation of non-specific precipitate, which could not be eliminated in the case of the fresh normal sera by varying the concentration of the saline in which the reaction was carried out.

It is difficult to argue from our evidence that a specific reaction between the antiserum and homologous antigen takes place, but taken in conjunction with the evidence from complement fixation tests, we may state that it appears likely to be the case.

Globulin derivatives: the significance of the carbobenzyloxy group

Bulk reactions set up between G.C.T. globulin, G.T. globulin and the antisera to them occurred in an extended zone of precipitation with an optimum at antigen dilution 1/13,000 in each case, thereby bearing out the experience of Clutton *et al.* [1938] with the ring test. When the corresponding derivatives of rabbit serum proteins were used as test antigens, similar results were obtained except that there appeared in each case to be a zone of non-specific precipitation (shown by normal sera also to a lesser degree) up to antigen dilution 1/600, and a second extended zone of less copious precipitation with optima about 1/38,000.

The differences between the sera did not justify any conclusions concerning the effect of the carbobenzyloxy group, and recourse was therefore had to inhibition reactions.

Inhibition reactions. Various possible inhibitors were tried at pH 7.0 using $M/25$, $M/250$ and M/∞ concentrations in saline. 0.25 ml. of inhibitor was incubated with 0.25 ml. of serum (1 in 3) for 2 hr. at 37°; 0.5 ml. of the rabbit serum protein derivative was then added in a concentration serologically equivalent to the antiserum. Equivalence was previously determined by the optimal proportions method of Dean & Webb [1926]. The results after further incubation are shown in Table II.

Table II. *Inhibition of precipitation between antisera to globulin derivatives and similar rabbit serum derivatives*

Antigens	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.
Anti-G.C.T. globulin	—	—	I	i + + +	—	i	I + + +	I + + +
Anti-G.T. globulin	—	—	i + +	i + +	i +	i +	I + +	I + + +
Inhibitors	Tyrosine ethyl ester		N-Carbobenzyl-oxytyrosine methyl ester		N-Acetyltyrosine hydrazide		O-β-Glucosido-N-acetyltyrosine	
Antigens	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.		
Anti-G.C.T. globulin	I + + +	I + + +	i + + +	i + +	i + + +	i +		
Anti-G.T. globulin	I + + +	Not done	i +	—	i +	i +		
Inhibitors	O-β-Glucosido-N-carbobenzyl-oxytyrosyl hydrazide		N-Carbobenzyl-oxyglutamic acid		Glucose			

G.C.T. = G.C.T. rabbit serum. G.T. = G.T. rabbit serum.

I = Inhibition at inhibitor dilution $M/250$.

i = Inhibition at inhibitor dilution $M/25$.

(The + signs indicate degree of inhibition at the particular dilution. + + + = complete inhibition. I or i without a + sign = slight but definite inhibition.)

These results indicated that N-carbobenzyl-oxyglutamic acid (which contains only C. of the G.C.T. hapten group) could inhibit precipitation when, and only when, the C. group was involved. The results in the second column also suggested that the antiserum to G.T. globulin contained antibody to G.C.T. as well. This is a reasonable supposition in view of the mode of preparation of the antigen.

In order to confirm the effect of the carbobenzyloxy group, bulk precipitations were carried out with both sera and the homologous antigens over the whole range of precipitation, using as a check inhibitor G.C.T. hydrazide. The results are given in Table III, and represent 48 hr. readings. Control

Table III. *Anti-G.C.T. globulin serum against rabbit serum as antigen*

Inhibitor	Antigen dilution						
	1/2400	1/4800	1/9600	1/19,200	1/38,400	1/76,800	1/∞
Anti-G.C.T. globulin serum against rabbit serum as antigen							
None	+	+	+	+	+	±	—
G.C.T. hydrazide, 1%	±	±	—	—	—	—	—
C.-glutamic acid, 2%	±	+	+	±	±	—	—
Anti-G.T. globulin serum against G.T. rabbit serum as antigen							
None	+	+	+	+	±	±	—
G.C.T. hydrazide, 1%	—	—	—	—	—	—	—
C.-glutamic acid, 2%	+	+	±	+	±	±	—

(Controls were done on the sera and antigens being tested and on normal sera in the presence of the inhibitors.)

mixtures of inhibitor with antisera, normal sera and the antigen solutions remained clear.

The results show that although G.C.T. hydrazide will inhibit both reactions, *N*-carbobenzyloxyglutamic acid only inhibits (though not completely) the reaction between serum and antigen when the carbobenzyloxy group is involved; this group must therefore be significant as a hapten group.

Note on the physiological properties of O- β -glucosido-N-carbobenzyloxytyrosyl insulin

This compound retained a considerable degree of physiological activity. Thus, not only was it necessary to give casein, starch and glucose paste by a stomach tube to the rabbits before injections, but even with this precaution the rabbits occasionally went into convulsions 18–20 hr. after injection. With the kind help of Dr Kerr, a test of physiological activity was made on a starving rabbit. It received 0.065 mg. intravenously (equivalent to 1 unit of the original insulin on a *N*-basis), and the blood sugar fell from 115 to 82 mg./100 ml. during 40 min., taking 3 hr. to return to 105 mg./100 ml.

Having in mind the possibility that insulin with all, or nearly all of its "free" amino groups blocked might be resistant to digestion in the small intestine, one of us (J. H. H.), after fasting 12 hr., received 3.5 mg. of the insulin compound (equivalent to 50 units of the original insulin on a *N*-basis) by a duodenal tube, whose position was verified by X-ray. The blood sugar curve showed no fall during the next 2½ hr., but only a slight rise. Hence it seems that the compound is not absorbed intact.

DISCUSSION

Complement fixation tests with sera of relatively low antibody content are difficult owing to extraneous effects encountered, such as the occurrence of haemolytic antigen solutions and anticomplementary sera; with direct precipitation tests non-specific precipitates occur which are hard to eliminate and tend to obscure the results. While the work described above confirms the conclusions of Clutton *et al.* 1938 concerning the conversion of insulin and gelatin into full antigens by coupling with *O*- β -glucosido-*N*-carbobenzyloxytyrosine, it appears to contradict their suggestion that the carbobenzyloxy group is not antigenically significant. Only by assuming a hapten function for this group *per se* can we explain the fact that the reaction between *O*- β -glucosido-*N*-carbobenzyloxytyrosyl rabbit serum proteins and the antiserum to *O*- β -glucosido-*N*-carbobenzyloxytyrosyl horse globulin is partly inhibited by *N*-carbobenzyloxyglutamate, whereas with the corresponding derivatives from which the carbobenzyloxy group has been removed there is no inhibition. The evidence of complement fixation between *N*-carbobenzyloxytyrosyl gelatin and homologous antisera is of interest, since in this case gelatin has been converted into a full antigen without the introduction of a carbohydrate group. It is interesting that under the conditions which we have employed we, as others, have failed to obtain any clear evidence for antibodies to a gelatin derivative which will precipitate with the homologous antigen.

In the case of G.C.T. insulin the increased range of precipitation when homologous antiserum is used in place of normal serum is small, but suggestive of a true antigen-antibody effect. This, together with the evidence from complement fixation tests, indicates a probability that the substance can stimulate the formation of specific precipitating antibodies.

In view of a recent paper by Gaunt & Wormald [1939], where results were given showing that treatment of insulin with benzyl chloroformate robs it of its physiological activity, the fact that G.C.T. insulin is still active assumes an added interest, although its significance is at present only a matter for speculation.

SUMMARY

1. The techniques of complement fixation and bulk precipitation have been used to confirm and extend the observations of Clutton *et al.* on the immunological properties of the *O*- β -glucosido-*N*-carbobenzyloxytyrosyl derivatives of gelatin and insulin.

2. Evidence is given for the hapten function of the carbobenzyloxy group, and for the fact that *N*-carbobenzyloxytyrosyl gelatin behaves as a full antigen.

We wish to thank Prof. C. R. Harington for the hospitality of his department and for his kindness and encouragement, and we are grateful to Prof. A. A. Miles for his advice with the serological experiments. One of us (M. E. Y.) is in receipt of a personal grant from the Medical Research Council.

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CCXXXVIII. ON THE MECHANISM OF UREA FORMATION

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CURRENT views on the mechanism of urea formation are based on two outstanding discoveries, one due to Krebs & Henseleit [1932] and the other to Leuthardt [1938].

Krebs & Henseleit observed that ornithine and citrulline catalyse urea synthesis from NH_3 and energy-supplying substances such as lactic acid. They explained this catalytic effect by the assumption of a cyclic reaction leading from ornithine via citrulline and arginine to urea, and thus back to ornithine. The evidence for this suggestive theory consists in the demonstration of the catalytic effect of ornithine and citrulline. The intermediates, citrulline and arginine, have not yet been isolated. Leuthardt demonstrated urea formation from glutamine and NH_3 . The presence of energy-supplying substances was not necessary for this reaction. Furthermore, the synthesis was not catalysed by ornithine. Thus two different ways of urea formation seem to be possible.

The following experiments started with the investigation of the role of citrulline in the urea cycle of Krebs & Henseleit [1932] and led to observations which seem to connect the results obtained by the above workers with those of Leuthardt [1938].

Methods

Animals. Young rats, chiefly albinos, were used; all were males with an average weight of 200 g., normally fed on ordinary stock laboratory diet.

Slice technique. The liver was removed and slices were cut with a razor moistened in Ringer solution from time to time and then blotted. The slices were accumulated in a moist chamber. As they were not wetted with Ringer solution, they could be weighed and adjusted to 100 mg. wet weight.

In Exps. XIV–XXI, where unwashed tissue was used, the slices were placed, after weighing, in the vessels used for incubation. In all other experiments they were placed instead in beakers containing 20 ml. of Ringer solution, where they were left for 3 min., after which the fluid was decanted and the beakers refilled with fresh Ringer solution. This procedure was twice repeated, after which the slices were placed in the vessels.

Incubation of slices. Barcroft vessels were used fitted with a side-tube for irrigation with gas mixture. They contained in all experiments 4.9 ml. of fluid. The latter was NaHCO_3 -Ringer [Krebs & Henseleit, 1932] except for the experiments with phosphate buffer (Table II). The pH was 7.3. In experiments with added substrates, 0.5 ml. Ringer solution was replaced by 0.5 ml. of the respective substrate, brought to pH 7.3 before being added to the vessel content. The vessels were shaken in a water thermostat at 38° for 10 min. at a rate of 120 times per min. during which the Ringer-bicarbonate solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 . After equilibration the

taps were closed and the vessels shaken for the experimental period which included the period of equilibration.

Preparation for N estimations. After the period of incubation 4 ml. of the vessel contents were placed in a centrifuge tube graduated at 10 ml., followed by acidification with 1 ml. of 40 % trichloroacetic acid and centrifuging. The fluid was neutralized with NaOH to pH 6.5, and made up to mark (solution A). 3 ml. were used for NH_3 estimation. The remainder of the fluid was treated with urease and incubated at 38° for 2 hr. (solution B). 3 ml. of solution B were then used for the estimation of $\text{NH}_3\text{-N} + \text{urea-N}$. 3 ml. of the remaining solution were placed in a tube graduated at 10 ml., H_2SO_4 (final concentration 5 %) was added and the fluid was then heated for 7 min. on a boiling water bath, neutralized and made up to mark (solution C). 5 ml. of solution C were used for the estimation of amide-N. To 4 ml. of the remaining solution C, 10 drops of 20 % $\text{Mg}(\text{OH})_2$ suspension were added, and the mixture was placed on the boiling water bath for 25 min. The fluid after acidification with a few drops of glacial acetic acid was then used for amino-N estimation. When amide-N was not estimated the treatment with H_2SO_4 was omitted. The latter did not affect the results of amino-N estimation.

Estimation of NH_3 , urea and amide-N. The NH_3 estimation was carried out by distillation according to Parnas & Heller [1924] with collection in 0.01 N HCl and titration with 0.01 N NaOH using a microburette. Solution A gave the $\text{NH}_3\text{-N}$ content, B minus A the urea-N content and C minus B the amide-N content. Recovery experiments for $\text{NH}_3\text{-N}$, urea-N and amide-N yielded 97, 99.5 and 92 % respectively of the theoretical values.

Estimation of amino-N. The amino-N was estimated with the Van Slyke apparatus. The amino-N obtained in recovery experiments with arginine represented 88 % of its α -amino-N; 85 % of the two amino-N atoms were recovered from ornithine [cf. Edlbacher & Burchard, 1931]. 110 % of an amount equivalent to one amino-N per mol. were recovered in amino-N estimations with citrulline. The yields given above were obtained in recovery experiments in presence of tissue and after the preparatory treatment of the amino-acid solutions as described earlier.

Reagents. *dl*-Citrulline was prepared in the following way: arginine flavianate was prepared from gelatin, from the former benzyldenearginine was obtained and finally arginine HCl [Whitmore, 1932]. Citrulline was made from arginine HCl according to the method of Fox [1938].

In the later experiments a preparation of *dl*-citrulline was used which was kindly supplied by Bayer Products Ltd., London, W.C. 2, to whom I wish to express my gratitude. Further, I am greatly indebted to Dr R. Duschinsky, Hoffmann La-Roche & Co., Fontenay-Sous-Bois (Seine) for a gift of 1 g. *l*(+)-citrulline, to Mr S. Williamson for a preparation of α -ketoglutaric acid, to Mr R. L. M. Syngé for a preparation of glutamine, and finally to Dr L. Zervas for a gift of *l*(+)-arginine, *l*(+)-ornithine and *l*(+)-glutamic acid. The three last-named preparations were the products of Hoffmann La-Roche & Co., Basle. All the other reagents were obtained from British Drug Houses Ltd.

EXPERIMENTAL

According to Krebs & Henseleit [1932] ornithine and citrulline, when added to liver slices of rat in presence of NH_4Cl and lactate, give rise to urea formation and cause partial disappearance of NH_3 . The experiments in Table I were carried out with citrulline, for which only few experimental results were given by the earlier workers.

Table I. *The effects of ammonium lactate and dl-citrulline on urea formation*Exp. period: 90 min. Final concentrations of substances added: *dl*-citrulline, 0.2%; lactate, 0.2%; NH_3 , 0.012%

No. of exp.	Substances added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		A Change $\text{NH}_3\text{-N}$ $\mu\text{g.}$	B Change urea-N $\mu\text{g.}$	Ratio $\frac{A}{B}$
		Before incubation	After incubation	Before incubation	After incubation			
X	None	20	0	0	10	- 20	+ 10	—
	<i>dl</i> -Citrulline	20	0	10	10	- 20	0	—
	Ammonium lactate	430	310	20	140	- 120	+ 120	—
	<i>dl</i> -Citrulline + ammonium lactate	390	210	110	285	- 180	+ 175	1.03
I	None	37	37	28	47	0	+ 19	—
	Citrulline	32	32	33	52	0	+ 19	—
	Ammonium lactate	455	325	0	159	- 130	+ 159	—
	<i>dl</i> -Citrulline + ammonium lactate	473	260	11	200	- 213	+ 189	1.12
III	None	0	0	14	33	0	+ 19	—
	Citrulline	0	0	14	47	0	+ 33	—
	Ammonium lactate	440	300	0	80	- 140	+ 80	—
	<i>dl</i> -Citrulline + ammonium lactate	430	130	10	310	- 300	+ 300	1.0

The results of Table I confirm the effect of citrulline on urea formation observed by these workers inasmuch as urea synthesis was markedly increased by citrulline in presence of ammonium lactate. The ratio of $\text{NH}_3\text{-N}$ disappearing (A) to urea-N formed (B), on the other hand, was approximately 1 in all cases, whereas it should be 0.5 according to Krebs & Henseleit [1932] if urea formation from citrulline were necessarily preceded by arginine synthesis, in which 1 atom of $\text{NH}_3\text{-N}$ is needed for every 2 atoms of urea-N formed. Further results in Table II showing amino-N determinations performed simultaneously with the experiments of Table I demonstrate that no significant changes in amino-N occurred during urea synthesis in presence of citrulline, whereas an increase in amino-N, owing to formation of ornithine (see methods), would be expected according to Krebs & Henseleit [1932]. When however arginine was used as a catalyst, amino-N markedly increased.

Table II. *Amino-N changes during urea synthesis catalysed by citrulline or arginine*Exp. period: 90 min. Final concentrations of substances added: *dl*-citrulline, 0.2%; α -ketoglutaric acid, 0.2%; *l*(+)arginine, 0.1%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. amino-N}$			$\mu\text{g. urea-N}$ formed
		Before	After	Change	
I	<i>dl</i> -Citrulline + ammonium lactate	950	961	+ 11	189
II	" "	922	945	+ 23	240
IV	" "	920	905	- 15	200
XVI	<i>dl</i> -Citrulline + α -ketoglutaric acid + NH_4Cl	985	920	- 65	170
XX	<i>l</i> (+)arginine + α -ketoglutaric acid + NH_4Cl	354	554	+ 200	550

Further, it will be seen from Table I that only insignificant amounts of urea were formed when citrulline alone was added to the washed tissue. In some cases, on the other hand, there was a definite synthesis when ammonium lactate

was added in the absence of citrulline. The addition of liver *Kochsaft* and liver extract from amounts of tissue greater than those used in the experiments failed to increase urea formation to the level obtained in the presence of citrulline (Table III), thus suggesting that urea formation from ammonium lactate is not determined by water-soluble or heat-stable constituents of the tissue such as citrulline.

Table III. *The effects of liver Kochsaft and liver extract on urea formation from ammonium lactate*

Exp. period: 90 min. Final concentrations of substances added: as in Table I.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. urea-N}$			Effect of citrulline	Effects of liver <i>Kochsaft</i> and liver extract
		Before	After	Change		
III	Ammonium lactate	0	80	+ 80	—	—
	Ammonium lactate + <i>dl</i> -citrulline	10	310	+ 300	+ 220	—
II	Ammonium lactate	0	146	+ 146	—	—
	Ammonium lactate + <i>dl</i> -citrulline	0	240	+ 240	+ 94	—
VI	Ammonium lactate	0	72	+ 72	—	—
	Ammonium lactate + liver <i>Kochsaft</i> *	11	103	+ 91	—	+ 19
VIII	Ammonium lactate	0	52	+ 52	—	—
	Ammonium lactate + liver extract†	83	158	+ 75	—	+ 23

* Liver *Kochsaft*: 1 g. of minced liver boiled in 1.6 ml. Ringer sol. for 7 min., 0.3 ml. of centrifugate added.

† Liver extract: 2.5 g. minced liver ground with sand and 2.5 ml. Ringer sol., centrifuged and 0.3 ml. of centrifugate added.

As the effect of added lactate was found not to be very consistent, the question arose as to whether substances derived from lactate, such as keto-acids, might play an active part in the formation of urea in the absence of citrulline. α -ketoglutaric acid was chosen for the following experiments.

Table IV. *Effects of α -ketoglutaric acid, NH_4Cl and *dl*-citrulline on urea synthesis*

Exp. period: 90 min. Final concentrations of substances added: *dl*-citrulline, 0.2 %; α -ketoglutaric acid, 0.20 %; NH_3 , 0.012 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$		Ratio $\frac{A}{B}$
		Before	After	Before	After	$\text{NH}_3\text{-N (A)}$	Urea-N (B)	
XVIII	α -Ketoglutaric acid	0	40	0	0	+ 40	0	—
XIII	NH_4Cl	490	482	18	0	- 8	- 18	—
XI	NH_4Cl	460	440	30	42	- 20	+ 12	—
XIV	NH_4Cl + ketoglutaric	490	347	0	118	- 143	+ 118	—
XVII	" "	486	328	27	123	- 158	+ 96	—
XI	" "	480	366	0	74	- 114	+ 74	—
XX	NH_4Cl + ketoglutaric + citrulline	503	254	9	266	- 249	+ 257	0.97
XII	NH_4Cl + ketoglutaric	508	399	0	79	- 109	+ 79	—
	NH_4Cl + ketoglutaric + citrulline	508	324	0	166	- 184	+ 166	1.11

It will be seen from Table IV that urea formation took place regularly in the washed tissue in presence of α -ketoglutaric acid + NH_3 but in absence of citrulline. Both NH_3 disappearance and urea formation were of about the same

order as when ammonium lactate was added (Table I), and both phenomena were intensified by the addition of citrulline to a similar extent as was earlier observed in presence of ammonium lactate (Table I). No significant N changes were observed with either NH_4Cl alone, or with α -ketoglutaric acid alone. Again, the quotient $\frac{\text{NH}_3\text{-N disappeared}}{\text{Urea-N formed}}$ was found to be approximately 1 when citrulline was added.

The important role of α -ketonic acids was further shown by experiments on urea formation from lactate and ketoglutaric acid in presence of alanine and glycine. Since glycine condenses with ketonic acids [Bach, 1939] an inhibiting effect of glycine on urea formation was to be expected in presence of ketoglutaric acid. Table V demonstrates this inhibition and a similar effect of *dl*-alanine.

Table V. *The effects of dl-alanine and glycine on urea formation from α -ketoglutaric acid + NH_4Cl*

Exp. period: 90 min. Final concentrations of substances added: α -ketoglutaric acid, 0.2%; NH_3 , 0.012%; *dl*-alanine and glycine, 0.2%.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amino-N
XVI	α -Ketoglutaric acid + NH_4Cl	495	345	0	115	- 150	+ 115	+ 50
	α -Ketoglutaric acid + NH_4Cl + glycine	495	447	25	13	- 48	- 12	- 230
XIV	α -Ketoglutaric acid + NH_4Cl	490	347	0	118	- 143	+ 118	+ 140
	α -Ketoglutaric acid + NH_4Cl + <i>dl</i> -alanine	490	410	0	55	- 80	+ 55	- 170
	<i>dl</i> -Alanine	0	18	0	116	+ 18	+ 116	- 120
XV	<i>dl</i> -Alanine	0	0	18	83	0	+ 65	—
	<i>dl</i> -Alanine + α -ketoglutaric acid + glycine	9	9	9	62	0	+ 53	—

Exp. XVI in Table V shows urea formation and NH_3 disappearance of the same order as usually obtained from ketoglutaric acid and ammonia, while the appearance of $\text{NH}_2\text{-N}$ suggested amination of ketoglutaric acid to glutamic acid. Urea formation was entirely inhibited when glycine was added, while a considerable disappearance of amino-N indicated condensation. *dl*-Alanine, which, unlike glycine, is known to give rise to urea in liver slices [Krebs, 1933; Bach & Holmes, 1937] had a similar effect though to a smaller extent, urea formation being only partly inhibited from both ammonium lactate and ketoglutaric acid and NH_3 . Again amino-N disappeared. If, on the other hand, *dl*-alanine was added alone, urea was synthesized and the disappearance of amino-N indicated deamination. If glycine was added, however, to alanine and ketoglutaric acid the latter seemed to react preferentially with glycine, thus preventing the removal of the alanine by the ketoglutaric acid. The effect of the keto-group in the mechanism of urea formation is thus evident.

The following experiments were devoted to the question as to whether ketoglutaric acid or pyruvic acid exerted an effect on citrulline in absence of NH_3 .

Table VI demonstrates urea formation in five successive cases from citrulline in presence of ketoglutaric acid or pyruvic acid, but in absence of added NH_3 .

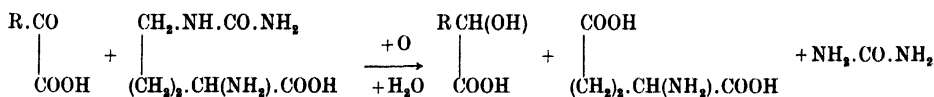
Table VI. *The effects of α -ketoglutaric acid and pyruvate on urea formation from citrulline in absence of ammonia*

Exp. period: 90 min. Final concentrations of substances added: *l*(+)-citrulline, 0.1 %; pyruvate, α -ketoglutaric acid and *dl*-citrulline, 0.2 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$	
		Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N
XXII	<i>l</i> (+)-Citrulline	0	0	0	0	0	0
	<i>l</i> (+)-Citrulline + α -keto-glutaric acid	0	0	0	44	0	+44
	<i>l</i> (+)-Citrulline + pyruvate	0	0	0	53	0	+53
XVI	<i>dl</i> -Citrulline + α -keto-glutaric acid	0	9	0	80	+ 9	+ 80
XVIII	<i>dl</i> -Citrulline	0	0	0	9	0	+ 9
	<i>dl</i> -Citrulline + α -keto-glutaric acid	0	0	0	53	0	+53
XXIII*	None	0	12	35	23	+12	-12
	Pyruvate	0	0	35	35	0	0
	<i>l</i> (+)-Citrulline + pyruvate	0	0	35	130	0	+95

* 500 mg. liver slices used.

There was practically no NH_3 present in the tissue and only negligible changes in the NH_3 content were observed during the experimental period; there was no change in $\text{NH}_2\text{-N}$. Less urea was synthesized than in presence of NH_3 . In view of the absence of other nitrogenous substances and the unchanged $\text{NH}_2\text{-N}$ it can be concluded that the urea formed originated from the citrulline itself. As in absence of ketoglutaric acid citrulline gives rise to only insignificant amounts of urea, while on the other hand considerable amounts of urea are formed in the presence of a keto-acid the role of α -ketonic acids in this reaction had to be explained. The following experiments were based on the hypothesis that ketonic acids might act as oxidizing agents inducing oxidation of the citrulline, followed by hydrolysis to glutamic acid and urea. This mechanism may be pictured as follows:



The role of glutamic acid in urea synthesis was, therefore, next investigated.

Table VII. *The effect of glutamic acid on urea formation*

Exp. period: 90 min. Final concentrations of substances added: *dl*-glutamic acid, α -ketoglutaric acid, 0.2 %; NH_3 , 0.012 %; *l*(+)-glutamic acid, 0.1 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. changes in}$			
		Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	$\text{NH}_2\text{-N}$	Amide-N
XVI	<i>dl</i> -Glutamic acid	0	0	0	0	0	0	-20	—
XVII	<i>l</i> -Glutamic acid	0	0	26	26	0	0	—	—
XVIII	<i>dl</i> -Glutamic acid + keto-glutaric acid + NH_4Cl	486	363	27	88	-123	+ 61	-60	—
	<i>l</i> (+)-Glutamic acid + keto-glutaric acid + NH_4Cl	486	310	27	97	-176	+ 70	0	—
XXI	<i>l</i> (+)-Glutamic acid + NH_4Cl	525	387	0	100	-138	+100	0	+40

The results given in Table VII show that no urea was formed if either *l*(+) or *dl*-glutamic acid was added alone, but considerable amounts were synthesized when NH_3 was also added. The addition of ketoglutaric acid also resulted in a smaller urea synthesis than was found in the absence of the keto-acid. The amino-N content remained unchanged when *l*-glutamic acid was used while a small disappearance was observed in two cases where *dl*-glutamic acid was added.

The fact that urea was formed from glutamic acid in presence of NH_3 suggested a possible formation of glutamine, especially as Exp. XXI of Table VII revealed the formation of amide-N. The action of glutamine was therefore tested.

Table VIII. *The effect of glutamine on urea formation*

Exp. period: 90 min. Final concentrations of substances added: glutamine, 0.1 %; α -ketoglutaric acid, 0.2 %; NH_3 , 0.012 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amide-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amide-N
XVIII	Glutamine	—	—	8	53	—	—	—	+ 45	—
	Glutamine + keto-glutaric acid	—	—	8	52	—	—	—	+ 44	—
XIX	Glutamine + NH_4Cl	550	486	0	144	450	410	- 64	+144	- 40
	Glutamine + NH_4Cl + ketoglutaric acid	514	363	53	167	430	315	- 151	+114	-115
	Ketoglutaric acid + NH_4Cl	505	407	17	79	46	82	- 98	+ 62	+ 36

According to Table VIII glutamine when added alone to the liver slices gave rise to only small amounts of urea, possibly owing to hydrolysis of the amide, while the addition of NH_3 to glutamine increased the yield by 200 %. An appearance of small amounts of amide-N from ketoglutaric acid and NH_3 indicated glutamine formation which in turn could explain urea synthesis from ketoglutaric acid and NH_4Cl in absence of other urea precursors.

As glutamine is known to be partly converted into α -pyrrolidonecarboxylic acid [Chibnall & Westall, 1932] the estimated values for ammonia-N, urea-N and amide-N could not be expected to balance as some of the amide- or amino-N may have escaped estimation.

The reactions here proposed for citrulline should equally apply to ornithine except for the initial stage of oxidation in presence of keto-acid, by means of which ornithine should be oxidized to glutamine.

Evidence for this concept is produced in Exp. XXII of Table IX in which small amounts of amide-N were formed from ornithine and ketoglutaric acid, while practically no urea appeared. When the experiment was carried out, however, in presence of ammonium lactate approximately 160 $\mu\text{g.}$ of urea were formed as compared with an average formation of approximately 250 $\mu\text{g.}$ from citrulline according to Table I. The difference can be explained. Citrulline and ornithine both give rise to glutamine which acts as catalyst in the formation of urea. In addition to this effect, citrulline yields urea by oxidation and hydrolysis. The amount is of the order of 60 $\mu\text{g.}$ according to Table VI. Although in the experiments with ornithine and ketoglutaric acid (Table IX) no trace of ammonia could be detected, the possibility remains that the glutamine found might have been derived from ketoglutaric acid and an unknown ammonia source.

Table IX. *Effects of ketoglutaric acid and ammonium lactate on urea formation from ornithine*

Exp. period: 90 min. Final concentrations of substances added: ketoglutaric acid, 0.2%; ammonium lactate, 0.2%.

No. of exp.	Subs. added	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amide-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amide-N
XXII	<i>l</i> -Ornithine 0.1 %	0	0	0	8	0	0	0	8	0
	<i>l</i> -Ornithine 0.1 % + pyruvate	0	0	0	0	0	0	0	0	0
	Ornithine 0.1 % + ketoglutaric acid	0	0	0	10	0	23	0	+ 10	+ 23
V	None	0	0	42	105	—	—	0	+ 63	—
	<i>dl</i> -Ornithine 0.2 %	0	0	63	94	—	—	0	+ 31	—
	<i>dl</i> -Ornithine 0.1 %	0	0	42	94	—	—	0	+ 52	—
	Ammonium lactate	450	334	85	128	—	—	- 116	+ 43	—
	Ammonium lactate + 0.2 % ornithine	460	220	95	253	—	—	- 240	+ 158	—
	Ammonium lactate + 0.1 % ornithine	530	270	0	161	—	—	- 260	+ 161	—

Table X. *Urea formation from ornithine, citrulline and arginine*Exp. period: 90 min. Final concentrations of substances added: amino-acids, 0.1%; ketoglutaric acid, 0.2%; NH_3 , 0.012 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amino-N}$		$\mu\text{g. changes in}$		
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amino-N
XX	Ketoglutaric acid + NH_4Cl + <i>l</i> -ornithine	484	262	54	233	580	580	- 222	+ 169	0
	Ketoglutaric acid + <i>l</i> -citrulline + NH_4Cl	503	254	9	266	483	548	- 249	+ 257	+ 65
	Ketoglutaric acid + NH_4Cl + <i>l</i> -arginine	503	272	67	621	354	554	- 231	+ 554	+ 200

Table X presents a comparison of the effects of *l*-ornithine, *l*-citrulline and *l*-arginine on urea formation in presence of ketoglutaric acid and NH_4Cl . Urea formation from arginine takes place about three times as fast as from ornithine and about 1.5 times as fast as from citrulline. The amino-N is only little increased with citrulline, remains unchanged with ornithine and is markedly increased with arginine. Finally, the NH_3 consumption is approximately the same in all three cases.

The changes in amide-N observed in Tables VII, VIII and XI led to the assumption of a continuous synthesis and breakdown of the amide group and strengthened the idea that glutamine, like ornithine and citrulline, may act as a catalyst for urea synthesis. It was therefore necessary to reinvestigate the role of CO_2 and CO_2 precursors as a source of carbon for urea formation. Preliminary experiments in this direction are recorded in Table XI which shows a marked increase in urea formation from *l*-glutamic acid if Na formate + NH_4Cl were added. A full N-balance sheet is given in Exp. XXI, Table XI, showing that NH_3 was used both to increase amide-N and urea, while amino-N remained unaltered. Owing to the difficulty caused by the formation of pyrrolidone-carboxylic acid from glutamine, a similar balance could not be set up in Exp. XXIV. This experiment, however, revealed again a very marked increase in urea formation from glutamine + NH_4Cl when Na formate was added to the

Table XI. *The effect of Na formate on urea formation from glutamic acid and glutamine*

Exp. period: 90 min. Final concentrations of substances added: *l*-glutamic acid, 0.1 %; glutamine, 0.1 %; Na formate, 0.1 %.

No. of exp.	Subs. added to 100 mg. liver slices	$\mu\text{g. NH}_3\text{-N}$		$\mu\text{g. urea-N}$		$\mu\text{g. amide-N}$		$\mu\text{g. changes in}$			
		Before	After	Before	After	Before	After	$\text{NH}_3\text{-N}$	Urea-N	Amide-N	Amino-N
<i>Unwashed tissue</i>											
XXI	<i>l</i> -Glutamic acid + NH_4Cl	525	387	0	100	35	75	- 138	+ 100	+ 40	0
	<i>l</i> -Glutamic acid + NH_4Cl + Na formate	525	343	0	147	35	68	- 182	+ 147	+ 33	0
<i>Washed tissue</i>											
XXIV	Glutamic + NH_4Cl (NaHCO_3 buffer)	575	648	10	12	—	—	+ 73	+ 2	—	—
	Glutamic + NH_4Cl + Na formate (NaHCO_3 buffer)	575	531	10	181	—	—	- 56	+ 171	—	—
	Glutamic + NH_4Cl (phosphate buffer)	575	637	10	42	—	—	- 62	+ 32	—	—
	Glutamic + Na formate	575	478	10	252	—	—	- 97	+ 242	—	—

washed tissue. The urea synthesis was even greater when the experiment was carried out in phosphate buffer, probably indicating that bicarbonate was not a necessary substrate for the synthesis while Na formate seemed to serve this purpose.

DISCUSSION

In the experimental part the suggestion was tentatively put forward as a working hypothesis, that citrulline forms urea in two stages. The first can be described as oxidative hydrolysis, giving glutamic acid and urea; in the second the product of oxidation, glutamic acid, forms glutamine which in turn gives urea in presence of NH_3 and of CO_2 or a precursor of CO_2 .

The evidence for the first is based mainly on the experiments recorded in Table VI, showing the formation of urea in presence of a ketonic acid and in absence of NH_3 . Whether the ketonic acid is the H acceptor in this reaction, as suggested, or whether the oxidation of the ketonic acid in turn induces the oxidation of the terminal CH_2 -group of citrulline, has not been investigated so far. No direct proof is at present available for the assumed formation of glutamic acid or of glutamine arising from this reaction. An alternative explanation can be based on the results of Ikeda [1938], who found in perfusion experiments with citrulline in dog's liver that 136.8 mg. citrulline had disappeared from 189.9 mg. citrulline added to the perfused blood, whilst urea and ornithine were formed in considerable quantities. In the experiments here described with liver slices of rats however no ornithine was found as shown in Table II.

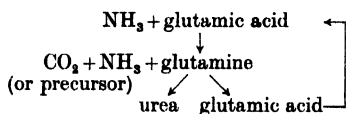
That ketoglutaric acid acts as " NH_3 acceptor" has been brought to light by the work of Braunstein & Kritzmann [1937] and of Kritzmann [1939]. It can therefore be assumed that under the experimental conditions the formation of urea from ketonic acids such as ketoglutaric acid in presence of NH_3 proceeds through glutamic acid, which in turn was shown to yield urea in presence of NH_3 .

Thus, in these experiments, the part played by the ketonic acid which is necessary for the formation of urea from citrulline in the absence of NH_3 does

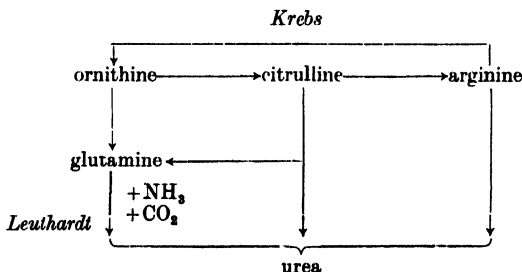
not appear to be merely that of a source of energy, as assumed by previous workers, but may be twofold. In the first place, in the presence of citrulline or ornithine, it may act as an oxidizing agent, as suggested above. For instance, in the case of ornithine, small amounts of amide-N could be detected, which according to the analytical methods applied must be rightly ascribed to glutamine. Secondly the ketonic acid behaves as a precursor of glutamine as shown by the rise in amino-N and the formation of amide-N in the experiments with NH_4Cl in the absence of amino-acids (Tables V and VIII).

The formation of glutamine from glutamic acid, as shown by Krebs [1935] and here assumed to occur in the course of the oxidative synthesis of urea, finds significant support in the experiments of Tables VII and XI, in which a clear N-balance sheet shows the formation of amide-N simultaneously with the urea. In these experiments almost 100% of the N-change could be accounted for in the fractions: NH_3 , amino-N, amide-N and urea-N.

The amide group thus appears to be essential for urea formation from glutamic acid and glutamine, as amide nitrogen is formed in presence of the former (Table VII) and partly disappears in presence of the latter (Table VIII). Further, urea synthesis fails to take place from glutamic acid in the absence of NH_3 . A cyclic formation and disappearance of amide-N from glutamic acid and NH_3 , which leads to the formation of urea, and which is set out below, appears to be an explanation.



The synthesis of urea from glutamine has been extensively proved by the work of Leuthardt [1938] who found that urea formation from glutamine was independent of the addition of "substrates for respiration" and of ornithine. The above investigations point to a possible connexion between the work of Krebs & Henseleit [1932] and that of Leuthardt [1938]. The following scheme gives expression to this view:



The experiments discussed above thus suggest a possibility of another pathway from ornithine and citrulline to urea in addition to that discovered by Krebs & Henseleit. The four different mechanisms may be pictured as follows:

1. The mechanism of Krebs.
2. The formation of urea from glutamine (Leuthardt).
3. Urea synthesis from citrulline by oxidative hydrolysis.
4. The possible oxidative conversion of ornithine and citrulline into glutamic acid and finally glutamine, where the pathway joins that suggested by Leuthardt.

SUMMARY

1. Citrulline, when added to liver slices, gives rise to urea in presence of ammonium lactate. The ratio $\text{NH}_3\text{-N}$ disappearance to urea-N formation was found to be approximately 1. Amino-N remained practically unchanged during the synthesis and was markedly increased when citrulline was replaced by arginine, showing that no ornithine was formed from citrulline. A marked urea formation was also obtained in presence of ammonium lactate but in the absence of citrulline or any other amino-acid.

2. When ammonium lactate was replaced by α -ketoglutaric acid and NH_4Cl the urea formed in absence of citrulline was accompanied by a rise in amino-N and of amide-N, interpreted as a synthesis of glutamine.

3. The role of ketonic acids in the mechanism of urea synthesis was further shown by experiments in presence of glycine and alanine. Urea formation from ketoglutaric acid and NH_4Cl was inhibited in presence of glycine and partially inhibited in presence of alanine, owing to the action of the amino-acids on α -ketoglutaric acid, during which amino-N disappeared.

4. A small but distinct synthesis of urea was observed in presence of citrulline and α -ketoglutaric acid or pyruvic acid in absence of NH_3 . This result suggested an oxidation of citrulline by the ketonic acids leading to the formation of urea and glutamic acid. A scheme for this reaction is outlined.

5. Glutamic acid and glutamine both give rise to urea formation in presence of NH_3 , but not in its absence. A satisfactory N-balance sheet was set up. It reveals a marked formation of glutamine from glutamic acid and a disappearance of amide-N from glutamine. These observations led to the concept of a cyclic formation and disappearance of glutamine, catalysing urea synthesis.

6. Ornithine, like citrulline, gives rise to urea formation in presence of α -ketoglutaric acid and NH_4Cl , but not in absence of the latter. This is in agreement with the hypothesis of its oxidation to glutamine which was supported by the appearance of amide-N, ascribed to glutamine according to the methods applied.

7. Urea synthesis from arginine in presence of ketoglutaric acid and NH_4Cl was found to be three times as fast, and from citrulline 1.5 times as fast, as from ornithine. Again, little change in amino-N was found with citrulline and none with ornithine whereas amino-N markedly increased with arginine. The disappearance of NH_3 was of about the same order in all three cases.

8. The role of CO_2 or CO_3 precursors as a source of carbon for urea formation was investigated. Na formate markedly increased urea formation in presence of NH_4Cl from glutamic acid or glutamine.

9. A scheme was outlined suggesting urea synthesis from ornithine, citrulline and also by hydrolysis from arginine, thus connecting the mechanism shown by Krebs [1933] with that observed by Leuthardt [1938].

I would like to express my thanks to Dr E. Friedmann for many inspiring and valuable discussions and also to Prof. Sir Frederick G. Hopkins for his interest and advice. I am indebted to Mr S. Williamson for carrying out the greater part of the amino-N determinations.

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CCXXIX. IRON, COPPER AND MANGANESE IN HUMAN ORGANS AT VARIOUS AGES

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THE usual statement in textbooks of physiology that the adult organism contains much less Fe than the young one is in noteworthy contradiction to a number of findings, some of them quite old. Lapique & Guillemonat [1889] and Lapique [1897] observed a characteristic life curve of the Fe content of blood-free human and animal livers and found higher values in the adult than in the child. The first part of this curve, however, shows a rapid drop from the high values of the newborn to the low level present in childhood. Physiologists seem to have concentrated their attention chiefly on this phenomenon and have thus often overlooked the later rise. More recently Lapique's results have been corroborated, in part at least, e.g. by Ramage *et al.* [1933] in the human, and by Lintzel & Radeff [1931] in several species of animals. One of us [Zondek & Karp, 1934] showed that in certain animals the Fe content of the organs rose considerably during a definite period of life. Since this increase occurred later in those animals whose span of life is longer, one could assume that there might exist a connexion between the ageing process of the cell and its Fe content.

In the present investigation we have extended our work to man: the literature contains little about the Fe content of human tissues and the figures found must sometimes be considered critically because of the methods used. Since, moreover, only the liver had been previously studied, we carried out a reinvestigation which included also the kidney.

In a number of cases Cu and Mn were also determined in order to show how far the life curve is typical for Fe and perhaps to contribute further information on the significance of the congenital Cu deposit.

EXPERIMENTAL

Non-haemin-Fe. An organ, e.g. liver, contains: (1) blood-Fe, of which 95–98 % is in the form of haemin-Fe (or more correctly: haem-Fe), accepting Barkan & Schales' [1937] hypothesis that there exists a small fraction of pseudo-haemoglobins; (2) tissue-Fe, of which 80–90 % occurs in the form of non-haemin-Fe whose chemical nature has not yet been defined, 10–20 % being haemin compounds such as cytochrome, catalase, respiratory enzyme etc. Although direct determinations for the latter group have been made only on the rat [Yabusoe, 1925], we may assume a similar distribution in human organs. Careful washing or perfusion of an organ can at best remove only the total blood-Fe. The residue thus contains not only non-haemin-Fe, as is usually believed, but also a certain amount of haemin-Fe, the cell catalysts mentioned above.

Here we were interested chiefly in the amount of non-haemin-Fe. A report of our experience with the various methods will be left to another paper; we

merely mention that we modified the original method of Tompsett [1935] in that we extracted the organs for a much longer time, since by the original method, only part of the non-haemin-Fe goes into solution. The same observation has been made in the meantime independently by Tingey [1937]. The method is as follows: the tissue is washed only slightly and minced finely with chrome-plated scissors; 1–2 g. of this substance are ground up in a mortar with glass powder; several ml. saturated $\text{Na}_4\text{P}_2\text{O}_7$ and 20 % trichloroacetic acid are added, the mixture is further ground up and allowed to stand for 24–48 hr. or, if the tissue contains very much Fe, for 3 days. The mixture is then transferred into a wide centrifuge tube with the aid of 10 % trichloroacetic acid and centrifuged; the residue is washed twice with pyrophosphate and trichloroacetic acid and the washings added to the first filtrate. Usually 5–10 ml. pyrophosphate and 10–20 ml. trichloroacetic acid were used.

Fe determinations were carried out on aliquot parts of the filtrate by the thiolacetic acid method [Hanzal, 1933; Burmester, 1934], and for control occasionally by the *o*-phenanthroline method (reducing agent: $\text{Na}_2\text{S}_2\text{O}_4$) or by the thiocyanate method (after ashing aliquot parts with H_2SO_4 and HClO_4 in order to convert the pyrophosphates into orthophosphates). All three methods gave the same results. The colorimetric estimations were performed with the very sensitive Leitz photometer, with filter 495 $\text{m}\mu$ for all three methods.

We felt it necessary to check our method of extraction with other methods, an obvious omission from previous work. The control methods were:

(1) Starkenstein & Weden's [1928] method, consisting in extraction of the non-haemin-Fe with 5 *N* HCl at 100°.

(2) Yabusoe's [1925] indirect method: the haemin-Fe is extracted with acidified methanol, the total Fe is determined in a second sample, and the non-haemin-Fe is calculated from the difference. It was found, however, that the direct colorimetric comparison of the haemin extracts, according to Yabusoe, with a standard haemin solution gave unreliable results, so that we ashed the extracts instead and determined their Fe content by the thiocyanate method of Kennedy [1927]. Table I shows the good agreement of the three methods and proves that all the non-haemin-Fe is actually extracted by pyrophosphate.

Table I. *Determination of non-haemin Fe with various methods*

(mg. per kg. dried tissue)

No.	Tissue	Tompsett method (modified)	Starkenstein method	Yabusoe (modified)
34	Kidney	175	—	163
35	Liver	—	1050	1140
61	Kidney	146	—	142
103	Liver	605	630	582
106	Liver	2400	—	2480
117	Kidney	230	—	257
143	Kidney	218	250	227

Our method, if applied to blood, extracts 2–3 % of the total Fe, corresponding to the findings of Tompsett [1934] and Shorland & Wall [1936]. Since in the organs examined, from which in no case had the blood been washed out, only about 30–60 % of the total Fe was in the form of blood Fe, the values for non-haemin-Fe can hardly be seriously influenced by that small fraction of "easily split-off" blood Fe (Barkan).

Total Fe. After determining the water content, dried samples of the tissue were ashed ($\text{H}_2\text{SO}_4 + \text{HClO}_4$) and the content of the ash solution determined by the thiocyanate method [Kennedy, 1927], occasionally also by the *o*-phenanthroline method [Heilmeyer & Plötner, 1937].

Copper. Aliquot parts of the ash solution were analysed by the usual sodium diethyldithiocarbamate method [McFarlane, 1932; Steussig, 1938]. We wish to mention that we were not always able to confirm the findings of McFarlane [1934] and Tompsett [1935] that the total Cu content of tissues is present in the trichloroacetic acid extract.

Manganese. With Mn, on the other hand, we found that treatment of the tissue for 24 hr. with 10 % trichloroacetic acid caused complete extraction of the metal (Table II). Thus we were able to simplify the procedure considerably

Table II. *Mn content of whole liver and CCl_3COOH extract*

No.	Total Mn	Mn in
		CCl_3COOH extract
35	6.5	6.1
81	10.0	9.2
108	8.2	8.7

by ashing merely the trichloroacetic acid extracts ($\text{H}_2\text{SO}_4 + \text{HClO}_4$) instead of the usual ashing of 5–10 g. whole tissue, which takes much time. The ashings were then transferred into a porcelain crucible, evaporated almost to dryness (sand bath), the residue dissolved in a few ml. hot 10 % H_2SO_4 and 5 drops *N*/10 AgNO_3 added. After centrifuging the Mn content of the filtrate was determined colorimetrically in the usual manner after oxidation to HMnO_4 with 1 ml. saturated $\text{K}_2\text{S}_2\text{O}_8$, using KMnO_4 as the standard solution. 1 μg . Mn in 3 ml. volume gave a sufficiently deep colour for colorimetric comparison. The optimal pH conditions have recently been carefully studied by Ray [1938] so that details need not be given here.

We need not emphasize that all the usual precautions were taken to prevent contamination and also in preparing standard solutions. The amount of the gravimetrically determined Fe standard was controlled also by titration. Blanks were run in every case. Recoveries were carried out for all methods and proved to be satisfactory.

Case reports. We used the current autopsy material of the hospital during the period extending from January 1938 to April 1939.¹ In almost every case the material was from immigrants of European origin, who had lived in Palestine for the most part from 1 to 10 years. Owing to the disturbances in this country, many of our cases included perfectly healthy individuals who had been killed by shooting.

In most cases the organs were analysed within 24 hr. after autopsy. We were able, however, to confirm the observations of Borgen & Elvehjem [1937] and Okamoto [1937], that storage at 4° up to as long as 3 days had no influence on the results.

RESULTS

In Table III the values for total Fe, non-haemin-Fe, and Cu in the livers and kidneys of a series of 75 cases are listed according to the age of the individuals.

¹ We are indebted to Dr H. Karplus for supplying us with the autopsy material.

Table III. *Fe and Cu content of liver and kidney*

(mg. per kg. dried tissue)

No.	Age	Sex	Diagnosis	Liver			Kidney			Remarks
				% water	Total Fe	Non-haemin Fe	% water	Total Fe	Non-haemin Fe	
116/17	Foetus (9½ months)	M.	Still-birth (asphyxia)	76.8	2750	2300	83.2	962	257	—
125/6	New-born	M.	Still-birth	79.7	3060	2900	84.7	339	175	—
114/15	New-born	M.	Died shortly after birth. Hyperaemia of brain	79.5	—	1550	84.2	538	168	—
119/20	New-born	M.	Died shortly after birth. Asphyxia	78.3	1340	1260	84.0	485	—	—
42/3	New-born	F.	Died shortly after birth. Asphyxia	82.0	590	490	83.9	441	—	—
40	2 days	F.	Microcephalus	—	—	—	83.5	370	220	14
49/50	3 "	F.	Pneumonia. Subdural haemorrhage	80.4	380	300	84.2	380	16	—
29/30	3 "	M.	Bronchopneumonia. Haemorrhage	—	2920	2500	—	504	37	—
138/9	3 "	M.	Pneumonia. Rupture of tentorium	74.0	—	1500	82.8	—	250	—
1/2	4 "	F.	Meningitis. Spina bifida	79.4	2800	2400	86.4	487	16	—
32/33	8 "	M.	Adrenal infarct	—	1315	1000	—	360	—	Fatty liver
108/9	9 "	M.	Pneumonia	77.5	—	2020	84.1	—	165	—
A/B	13 "	M.	Enteritis. Cystitis	76.5	3200	2900	82.3	1160	1000	420
143	13 "	F.	Enteritis. Congenital heart defect	—	—	—	84.3	444	214	—
122/3	14 "	M.	Melaena neonatorum. Pneumonia	81.0	2500	2450	85.6	266	210	Transfusion given
99/100	15 "	M.	?	77.5	—	1160	84.0	—	190	—
106/7	1½ months	M.	Pylorospasm. Pulmonary oedema	78.8	2720	2420	85.4	—	235	Fatty degener. of liver
90/1	3 "	M.	Ulcerative colitis	63.7	1000	700	82.4	—	134	—
110/11	4 "	M.	Enteritis. Nephritis. Anaemia. Haemorrhage	80.6	1050	1000	82.4	—	158	—
95	4 "	F.	Meningitis. Pyuria	—	—	—	85.4	—	104	—
132	5½ "	M.	Pneumonia	—	—	—	—	—	—	—
150/1	8 "	M.	Empyem. Abscess of lungs	77.0	—	250	83.0	—	85	—
101/2	12 "	F.	Enteritis. Anaemia	75.0	1250	800	83.5	—	98	Transfusions given
17	13 "	M.	Thrombosis of portal vein	—	—	—	84.0	175	63	—
133	15 "	F.	Empyem. Pericarditis	—	—	—	84.2	—	120	—
36/7	18 "	M.	Lymphat. Leukemia. Anaemia. Tumour spleen	74.2	240	140	81.3	150	60	18
67/8	20 "	F.	Pneumonia. Cretinism	73.5	690	450	79.4	—	70	—
92	21 "	M.	Sepsis	—	—	—	81.8	—	57	—
69/70	2 years	M.	Burn	75.6	250	63	81.2	—	53	Fatty degenerat. of liver, kidney
112/13	2 "	M.	Encephalitis	75.0	—	113	82.0	—	94	—
130/1	2½ "	F.	Heart block	77.5	—	90	83.4	—	90	—
83/4	4 "	F.	Fibr. Bronchitis. Glom. Nephritis	75.1	700	200	79.6	—	88	—

Fe, Cu AND Mn IN HUMAN ORGANS

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87/8	5	F.	Endocarditis	74.1	630	370	—	81.3	—	97	35	—
57	7	F.	Cardiac insufficiency	—	—	—	—	—	196	—	—	—
81/2	7	F.	Myocarditis. Oedema of lungs and liver	78.1	—	—	—	82.3	—	89	—	—
76	8	M.	Lymphosarcoma. Haemorrhage	77.0	—	350	—	—	—	—	—	—
134/5	12	M.	Aortic insufficiency	76.7	—	570	—	82.5	—	114	—	—
136/7	12	M.	Recurring endocarditis	76.0	—	600	—	79.5	—	125	—	—
10/11	16	M.	Drowning	—	900	—	55	—	456	—	18	—
54/5	17	M	Severe anaemia. Tumour of spleen. Bilharzia	76.4	1130	1040	16	83.0	220	203	11	—
141/2	17	M.	Abscesses. Embolism	75.7	—	370	—	80.0	—	102	—	—
89	19	F.	Drowning	—	—	—	—	81.3	262	112	—	—
60	22	F.	Pannyelephthisis. Anaemia	—	—	—	—	82.2	430	250	36	Transfusions given
62	22	M.	Fracture of skull	—	—	—	—	80.1	463	163	—	—
72	22	F.	Dysentery	—	—	—	—	84.9	—	140	—	—
26/7	23	M.	Duodenal ulcer. Anaemia. Nephritis. Endocarditis	74.7	198	—	25	85.0	370	230	30	—
144	24	F.	Accident. Asphyxia	77.4	—	620	—	—	—	—	—	—
64	24	M.	Shot	—	—	—	—	80.3	461	189	—	—
65	24	M.	Shot	—	—	—	—	83.0	390	140	—	—
38/9	26	F.	Peritonitis. Ileus	76.4	680	540	42	78.7	458	—	70	—
71	27	M.	Shot	—	—	—	—	78.4	—	170	—	—
63	28	F.	Endocarditis (recur.)	—	—	—	—	81.5	—	158	—	—
103/4	30	M.	Shot	70.8	700	590	—	81.5	292	110	—	—
118	32	M.	Osteomyelitis	79.4	2170	1240	—	—	—	—	—	—
22/3	34	M.	Veronal poisoning	75.7	1570	1240	22	83.0	540	—	26	—
58	35	M.	Shot	—	—	—	—	81.0	—	190	—	—
85/6	35	M.	Agranulocytosis. Icterus. Anaemia	76.0	2260	2000	—	81.8	278	210	—	Transfusions given
78/9	36	M.	Shot	74.2	1140	800	—	82.4	—	152	—	—
35	38	M.	Pulmonary tuberculosis	76.9	1250	1100	103	—	—	—	—	—
146/7	40	M.	Haemorrhage into brain. Hypertonia	72.0	—	186	36	76.5	720	256	39	Death after 2 hr.
59	40	M.	Pneumonia. Softening of the brain	—	—	—	—	80.4	409	173	—	—
73	40	M.	Shot	—	—	—	—	80.1	412	142	—	—
61	42	F.	Hanged	—	—	—	—	—	—	—	—	—
140	44	M.	Hanged	73.0	—	920	—	—	—	—	—	—
96/7	44	M.	Shot	74.8	1060	350	—	80.1	510	135	—	—
34	53	F.	Lysol poisoning	—	—	—	—	79.8	340	174	14	—
66	59	M.	Shot	—	—	—	—	83.9	—	173	—	—
105	60	M.	Tumour of lung. Sclerosis	77.1	—	1480	—	—	—	—	—	—
74/5	60	M.	Pneumonia. Laryngeal carcinoma	73.0	880	500	—	76.5	406	135	—	—
98	65	F.	Infarct of heart	76.7	985	350	—	—	—	—	—	—
145	65	M.	Infarct of heart	71.0	—	900	44	—	—	—	—	—
52	70	M.	Sepsis. Furunculosis	77.0	815	705	28	—	—	—	—	—
6/7	75	M.	Pneumonia. Fracture of skull	77.5	1080	1000	48	81.2	290	180	10	Death after 2 days
12/13	75	M.	Fracture of skull. Diabetes. Cardiac defect	—	2920	2500	34	—	410	—	42	—
148/9	84	M.	Severe anaemia. Carcinoma of stomach. Cardiac infarct	78.0	—	63	—	80.0	—	—	55	—

Iron. In spite of the fact that many of the cases were pathological and in spite of the great variability of the Fe content even in normal individuals, a typical distribution according to ages could be recognized. This is made even clearer by a study of the statistical evaluation of the figures for the various age groups (Table IV). The significance of this phenomenon will be discussed in detail later.

Table IV. *Non-haemin Fe in various age-groups*

Age	(mg. per kg. dried tissue)					
	Liver			Kidney		
	No. of cases	Mean value	Range	No. of cases	Mean value	Range
0-15 days	14	1770	300-2900	9*	205	165-257
3-12 months	4	690	250-1000	5	116	85-158
15 months-3 years	5	170	63- 450	7	78	53-120
4-12 years	5	350	200- 600	5	102	88-125
16-22 ..	2	705	370-1040	6	162	102-250
23-35 ..	6	1040	540-2000	8	175	110-230
36-75 ..	12	930	186-2500	10	160	84-256

* No. B not included.

Hitherto there were hardly any Fe values for perfectly healthy adults to be found in the literature, although Ramage *et al.* [1933] emphasized the importance of such figures some time ago. We present here the values for a series of selected cases without anatomical pathological findings, most of whom had been killed by shooting:

Liver. Total Fe (cases 103, 22, 96, 98, 6): average 1080 mg./kg. dried substance (range 700-1570).

Non-haemin-Fe (cases 144, 103, 22, 140, 96, 98, 145, 6): average 800 mg./kg. dried substance (range 550-1240).

Kidney. Total Fe (cases 62, 64, 65, 104, 23, 73, 61, 97, 34, 6): average 411 mg./kg. dried substance (range 292-540).

Non-haemin-Fe (cases 62, 64, 65, 71, 104, 58, 73, 61, 97, 34, 66, 7): average 161 mg./kg. dried substance (range 110-190).

For non-haemin-Fe, the ratio liver : kidney is 5 : 1. It is noteworthy that in various animal species different figures have been reported, e.g. in the rat 2 : 1.

The content of haemin-Fe, which is in the main dependent on the amount of blood present in the tissue examined, is about the same in liver and kidney, averaging 250-280 mg./kg. dried substance.

If one compares the average for non-haemin-Fe in the healthy individuals with the total average for all the cases (adults), there is practically no difference in the kidney. In the case of the liver, however, the total average is 20 % higher than the value found in healthy individuals, obviously owing to the fact that in a series of diseases there is a tendency for the Fe content of the liver to increase. (We shall refer later to the effect of blood transfusions.)

We were not able to observe definite differences due to sex either in the liver or kidney, in contradiction to the findings of Lapique & Guillemonat [1889]. For comparison with our absolute values, we quote the figures obtained by other authors, all in mg./kg. dried substance:¹ Lapique & Guillemonat [1889]: 1300 (new-born infants), 240 (1-2 years), 940 (male adults)—total Fe in blood-free liver; Hueck [1912]: 500 (adults, blood-free liver); Adler & Adler [1931]: 1330 (new-born infants), 447 (6 adults)—blood-free liver; Gladstone [1932]: 900 (9 newborns and infants)—non-haemoglobin-Fe in blood-containing liver;

¹ For calculation, the water contents of liver and kidney were taken as 75 and 80 %.

Toverud [1935]: 1800 (47 new-born infants)—blood-free liver. That Tompsett's [1935] values are lower because of the method used has already been mentioned. He reports 270 (12 adult livers) and 35 (12 adult kidneys). For total Fe in liver we quote Ramage *et al.* [1933]: 2250 (6 new-born infants), 800 (15 children between 6–12 years).

Copper. The existence of a congenital Cu deposit in the human liver is well known and has been confirmed by this investigation also. The average value for liver in 14 infants (0–1½ months) was 230 mg./kg. dried substance, ranging from 80 to 382. After the second month the values drop rapidly to the adult level, remaining more or less constant for the remainder of life.

Our average value for adults is 34.6 (12 cases, range 16–55). Most other investigators give similar figures, e.g. Ramage *et al.* [1933] 265 (24 new-born and infants up to 7 weeks, range 50–500), 60 (24 children from 3 to 12 years); Kleinmann & Klinke [1930]: 27.5 (adults); Herkel [1930]: 25.4 (10 adults); Tompsett [1935]: 22.0 (12 adults).

In one case (No. 35) with the diagnosis of tuberculosis, we found a definitely increased value. We should not consider this observation significant, were it not for the fact that similar findings were observed in the guinea-pig [Cherbuliez & Ansbacher, 1929]. We have further studied the figures compiled by Ramage *et al.* [1933] and found that 8 of a total of 12 cases with the diagnosis tuberculous meningitis showed a definite increase over the normal values of the corresponding ages. The significance of this phenomenon seems at present obscure.

There is no congenital Cu deposit in the kidney. The average for all ages is 26.3 (range 10–70). In comparison with Fe the individual variations are much greater, which may be due to the fact that the kidney is the excretory organ for Cu, but not for Fe. Case B (cystitis, enteritis) is unusual because of the extremely high value of 420. In the same case the Fe content of the kidney was increased fivefold so that there seems to have been a serious disturbance of the metal metabolism.

As Table V shows there is no congenital Cu deposit in the brain either, in contradiction to statements found at times in textbooks of biochemistry. Since there are considerable differences in the content of heavy metals in the various

Table V. *Cu content of brain*

(mg. per kg. dried tissue)		
No.	Age	Cu
121	New-born	15
46	New-born	20
124	14 days	6
16	13 months	14
9	16 years	50
56	17 „	23
80	36 „	35
5	75 „	32

parts of the brain, a more detailed study of this organ can be made only after careful anatomical dissection. Recently Tingey [1937; 1938] made an excellent study of the subject, and since his methods are almost identical with ours we can well compare his values with our figures for the remaining organs.

In addition, we made a few analyses, intended to be merely of an exploratory character, of several endocrine glands. We found surprisingly low Cu values for thymus, hypophysis and thyroid, between 4 and 20 mg./kg. dried substance. The Fe content of the same organs was between 100 and 300 mg./kg. dried substance.

Manganese. Table VI shows the Mn content of several livers. There seems to be no influence of age. The values are fairly constant, ranging between 4.5 and 10 mg./kg. dried substance, with the mean value of 7.0, identical with that of Richards [1930] and similar to the value 8.4 of Ramage *et al.* [1933] for 24 cases ranging in age from 3 to 12 years.

Table VI. *Mn content of liver*
(mg. per kg. dried tissue)

No.	Age	Mn
42	New-born	4.8
49	3 days	6.0
108	9 "	8.2
99	15 "	10.0
36	18 months	10.0
67	20 "	9.1
69	2 years	4.8
83	4 "	7.4
81	7 "	10.0
38	26 "	4.5
35	38 "	6.5
96	44 "	5.4
52	70 "	5.2

Several exploratory determinations for serum, for which 50–100 ml. serum had to be used, gave values from 1 to 3 μg per 100 ml., in accordance with Bertrand & Medigreceanu [1912] and in contradiction to the higher values of Reimann & Minot [1920].

DISCUSSION

Iron

As may be concluded from Tables III and IV, the typical life curve for total Fe and particularly for non-haemin-Fe is present not only in the liver, as was observed by Lapicque & Guillemonat [1889] and Ramage *et al.* [1933], but also in the kidney. In the latter organ the regularity is even more distinct, since the individual variations are fewer. What is the significance of this curve?

The values in the newborn are in general quite high, but there are exceptions. According to the classical theory [Bunge, 1898; Abderhalden, 1900] the newborn has a congenital reserve deposit of non-haemin-Fe, which is to compensate for the lack of Fe in the milk diet of the first few months. But a series of investigations indicate that this is true only in part [Schwartz *et al.* 1924; Adler & Adler, 1931; Gladstone, 1932; Ramage *et al.* 1933]. Some increase in non-haemin Fe occurs already during foetal life, but the bulk of it is not accumulated until after birth, when the well known post-natal blood destruction reduces the high haemoglobin values of the newborn to about 50 % within a few weeks, liberating large amounts of Fe.¹ This "mobilization of Fe metabolism" [Schwartz *et al.* 1924] is obviously dependent on the circumstances of birth, since the livers of infants born by Caesarian section and still-births, according to the authors quoted above, usually show low Fe values.

An interesting parallel to this are the recent observations of Horváth & Hollósi [1935] that the appearance of the polycythaemia neonatorum is also dependent on the circumstances of birth. Infants born by Caesarian section do not show an increased red cell count. Sachs *et al.* [1938], on the basis of similar findings, have suggested that the shock produced by the trauma of birth might

¹ Part of the liberated Fe is excreted, resulting in a negative Fe balance during the first 2 months [Stearns & McKinley, 1937].

lead to an increase in the erythrocytes. Obviously in the newborn large blood reserves can be put into circulation quite suddenly. Applied to our problem we must assume the following mechanism: birth \rightarrow shock \rightarrow polycythaemia \rightarrow blood destruction \rightarrow accumulation of the liberated Fe in the organs. Abnormal low Fe values, as in our cases Nos. 42 and 49, could thus be explained not only by Fe deficiency of the mother, but also on the basis of an absence of the shock reaction.

On the other hand one would expect low Fe values in every still-born infant. Yet our two cases (Nos. 116 and 125) concerned have unusually high values. Either the mechanism described is not correct, or it can occur in the uterus before birth, perhaps as the result of insufficient O_2 supply to the foetus. We intend to study this question further in the future.

The further course of the curve shows a definite decrease in the Fe values to a minimum which is reached at about 2 years. According to the classical theory mentioned above, the Fe reserves are called upon during lactation for the formation of blood and muscle haemoglobin, since mother's milk with its very low Fe content cannot satisfy the demands of the growing organism. We would expect, then, to find the lowest values after weaning, not at the age of 2 years, after the child has lived on a normal diet (rich in Fe) for some time. Ramage *et al.* [1933], who made the same observation, have previously called attention to this discrepancy.

It is similarly difficult to interpret the next part of the curve, a slow increase, more rapid after the 12th year of age, which reaches the final level of the adult between the 16th and 20th years. A similar rise in the Fe content of the organs of various animals was already reported in a previous publication [Zondek & Karp, 1934]. Since this rise appeared later in the species of animals whose span of life is longer, always in the middle period of life, it was suggested that there exists a relationship between the ageing process of the cell and its Fe content. In man the increase in the Fe values occurs much earlier, ending between the ages of 16 and 20, so that it is questionable whether it is based on the same phenomenon. But here too there is a definite period of life during which the increase occurs.

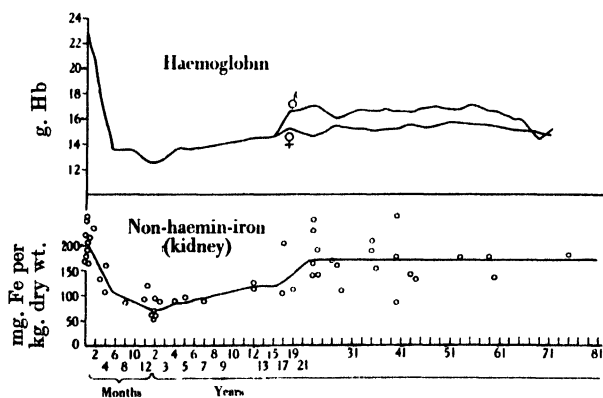


Fig. 1. Life-curves for the haemoglobin content of blood (Williamson [1916]) and the non-haemin Fe content of kidney.

The interpretation is made clearer by a comparison of our curve for non-haemin Fe with the familiar life curve for haemoglobin [Williamson, 1916] (Fig. 1). The course is so similar that we must assume a connexion. (The curve

for serum-Fe runs parallel in general, to judge by the few figures available for this Fe fraction during childhood [Thoenes & Aschaffenburg, 1934].)

We think it improbable that the so-called "physiological anaemia" of childhood can be explained by exogenous Fe deficiency. The diet of the normally fed child after weaning theoretically includes enough Fe to cover even the increased requirement of the growing organism. One might assume that the absorption of Fe is poorer than in the adult, but Hahn *et al.* [1938] were recently able to demonstrate in dogs by means of the radioactive indicator method ("labelled" Fe), that, particularly in Fe-deficient anaemias, the absorption of Fe, normally very slight, is greatly improved. The good effect of Fe therapy, so well known in such cases, need scarcely be mentioned. Further, if there were an extrinsic lack of Fe we might expect that the shape of the curve for non-haemin Fe, which chiefly represents the stored reserve Fe, would be a different one. In that case all the Fe reserves would be steadily used up for the formation of new haemoglobin, and the increase would not begin until after the cessation of growth, not at the end of the 2nd year as it actually does.

All of this points against Fe deficiency. If one does not wish to accept any other deficiency or a defect in the haematopoietic system during childhood, one is forced to the opinion that the young growing organism obviously requires less haemoglobin, and therefore less reserve Fe than the adult. During a certain period of development the requirement in both Fe fractions increases, a phenomenon which is reflected in the course of the two corresponding curves.

In the discussion of the adult values we do not wish to delve too much into the clinical details, but rather to confine ourselves to the following points. In the kidney we were unable to observe any differences due to sex. In so far as the liver is concerned, the three female cases were well under the average, but in so small a series it is impossible to draw any conclusions. We must therefore assume that the definite differences due to sex which Lapique & Guillemonat [1889] observed, were more conspicuous in his larger series.

The Fe content of the kidney is strikingly constant. Pathological influences are of hardly any consequence here, and in anaemias the Fe reserves of the liver seem to become exhausted much sooner than those of the kidney (case no. 26/27).

It was very interesting for us to observe how low the Fe content of the organs could be reduced as the result of severe anaemia or from other causes, considering the view of Hahn & Whipple [1936; 1938] that it is impossible to go below a certain minimum concentration. In the dog this "parenchyma-Fe" fraction which seems to be essential to life, is about 60 mg./kg. dried substance, of the same order as our minimum values in cases nos. 36/7, 69/70, 112/13, 130/1, 148/9. The last case demonstrates also that all the Fe acquired during growth can serve as reserve Fe in case of a severe anaemia.

Among the few factors which can definitely influence the Fe content we must above all mention blood transfusion. Gladstone [1932] showed in a detailed study that the destruction of the foreign blood which occurs in the reticulo-endothelial system promptly increases the Fe content of the spleen and later of the liver. Lapique [1897] made the same observation in animal experiments. As our cases nos. 101, 60, 85/6 show, we are in complete accord with these authors and can also include the kidney among the organs which are able to take up the Fe liberated by the destruction of the foreign red cells.

Copper

The catalytic influence of Cu on haemoglobin formation has now been conclusively proved for quite a series of animals [Elvehjem, 1935; Potter *et al.* 1938] and it seems probable that man belongs to this series. Cu influences the mobilization of the Fe deposits and their transformation into haemoglobin. It is generally believed that the congenital Cu deposit bears some relationship to this function. So far two hypotheses have been built up on this assumption: (1) Cu acts locally at the site of blood formation, which in the foetus is chiefly the liver; (2) Cu is stored in the foetal liver to compensate during the lactation period for the low Cu content of the milk. The shape of the Cu curve with its sudden decline after the second month is an argument against the latter point of view, because a more gradual decline during lactation should otherwise occur; but there are many possible objections to the first hypothesis as well. A comparative investigation of many animal species with regard to their congenital Cu and Fe deposits, foetal blood formation and the haemoglobin and Cu contents of the blood at birth would help to elucidate this question. So far preliminary studies of this kind have been made only by Cunningham [1931] and McFarlane & Milne [1934]. A further step toward the comprehension of the action of Cu has recently been made by the isolation of a Cu compound from liver and blood [Mann & Keilin, 1938].

Manganese

Whereas Mn seems to have an influence [Kemmerer *et al.* 1931; Daniels & Everson 1935] on the growth and sexual function of rats and mice, no function at all has been observed so far in man. But the Mn content of the liver (and of other organs) is so constant that it can hardly be considered an accidentally occurring element. The important discovery of Edlbacher & Pinösch [1937] and Edlbacher & Baur [1938] that almost all the Mn of the liver (cat, cattle) is found in the arginase extract of this organ and there represents the prosthetic group of that enzyme, points to a relationship between the Mn content and protein metabolism which it would be worth while to investigate further.

SUMMARY

1. After improving on certain methods, determinations were made of the content of total Fe, non-haemin-Fe, Cu and Mn of liver and kidney tissues at various ages. Occasionally some other organs were also examined.

2. In the case of liver and kidney, a characteristic life curve exists for the % of total Fe and particularly of non-haemin-Fe. Values are high at birth, then drop to a minimum which is reached at the age of 2 years, to rise again at first slowly, later more rapidly. From the age of 20 till the end of life, the values remain at the same level.

3. In the healthy adult the non-haemin-Fe contents of the liver and kidney are remarkably constant. The mean values are 800 and 160 mg./kg. dried substance respectively.

4. Severe anaemias may reduce the Fe content of the organs to a minimum which is identical with the minimal values of childhood and within the same limits as the "parenchyma-Fe" fraction found in the dog by Hahn & Whipple.

5. Blood transfusion is one of the few factors which definitely increase the non-haemin-Fe of both organs.

6. The life curve of the liver-Cu content shows the familiar maximum at birth, which is followed by a sharp decline after the second month. The mean

value for adults is 34.6 mg./kg. dried substance. The kidney and brain do not show maximum values at birth. The Cu content of several endocrine glands is surprisingly low. In a case of tuberculosis the Cu content of the liver was markedly increased, a phenomenon which seems to be of significance.

7. The Mn content of the liver is constant during the whole of life, 7.0 mg./kg. dried substance. The content of several sera is 1–3 μ g. per 100 ml.

8. The results are discussed in detail in the light of our present knowledge of the subject.

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CCXXX. FORMATION AND BREAKDOWN OF GLYCOGEN IN THE LIVER

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UNTIL a few years ago it was generally accepted that glycogen breakdown in animal tissues is initiated by an enzymic hydrolysis, giving glucose, or as some authors thought, a special "reactive form of hexose". Then Parnas & Baranowski [1936] discovered a new reaction of glycogen. They found that autolysed rabbit muscle extract forms from glycogen and phosphate a difficultly hydrolysable hexosemonophosphate which Ostern *et al.* [1936] showed to be the Embden equilibrium ester. This reaction, called by Parnas "phosphorolysis" of glycogen, differs from hydrolysis of glycogen in that phosphoric acid replaces water as the disruptive agent:

Hydrolysis of glycogen: $(C_6H_{10}O_5)_n + nH_2O = nC_6H_{12}O_6$.

Phosphorolysis of glycogen: $(C_6H_{10}O_5)_n + nH_3PO_4 = nC_6H_{11}O_6 \cdot PO_3H_2$.

The only other carbohydrate which reacts similarly in muscle extract is starch. Simple sugars and disaccharides are not phosphorylated. The extent of phosphorylation depends on the concentrations of glycogen and phosphate; with excess of phosphate present, all the glycogen is converted into hexosemonophosphate. With excess of glycogen, its breakdown is limited by the amount of phosphate available [Ostern *et al.* 1937].

Phosphorolysis seems to be the only method of breakdown of glycogen in muscle, and is the first step of the glycolytic cycle. A most important contribution to our knowledge of the mechanism of phosphorolysis was made by Cori *et al.* [1937], who showed that the hexosemonophosphate initially formed from glycogen is a new ester, glucose 1-phosphate (Cori ester), which is then converted into glucose 6-phosphate by the action of a second enzyme. Cori *et al.* [1938] further showed that this enzyme system also occurs in heart, brain, liver and yeast.

Liver extracts, dialysed for a short time, convert glycogen into Cori ester which is partly dephosphorylated to glucose. Since Davenport [1926] found that after removal of blood by perfusion little amylase could be found in liver, Cori & Cori [1938] suggested that glucose was formed in the liver by phosphorolysis rather than by the action of an amylase.

Later the presence of the phosphorylating enzyme in yeast was further confirmed by Schäffner [1939] and by Kiessling [1939], and Hanes [1939] has found in extracts of pea meal an enzyme which converts starch into Cori ester in the presence of inorganic phosphate.

Kiessling [1939] also showed that the reaction



is reversible. He isolated a protein fraction ("Protein C") from yeast which formed glycogen from Cori ester, and Cori ester from glycogen and phosphate, the same equilibrium being reached in both cases. An analogous reaction has very recently been described by Cori *et al.* [1939, 3] in muscle extracts from which

the enzyme converting the Cori ester into the Embden ester had been removed. From added Cori ester such extracts form glycogen which only differs from ordinary muscle glycogen in giving a blue colour with iodine.

In a preliminary note [Ostern & Holmes, 1939] we have reported a synthesis of glycogen from Cori ester in liver brei, and other experiments on the phosphorolysis of glycogen in liver. In the present paper we shall extend these findings and describe further experiments on the breakdown and synthesis of glycogen in the liver.

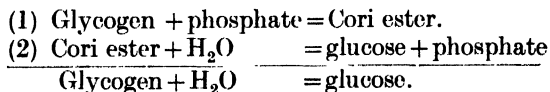
The material used has been mainly a concentrated (1/1) brei prepared from the livers of starved rabbits by mincing in a Latapie mincer. We found it very difficult to prepare liver extracts that would actively phosphorylate glycogen and such extracts were always very unstable. The use of a brei has the great advantage that the concentration relationships of the different enzymes are not disturbed, as they are in extracts, and the conditions more nearly approximate to those of the intact tissue.

If glycogen is added to such a brei, it is rapidly converted into glucose and the rate of breakdown is accelerated by phosphate; there is, however, no esterification of inorganic phosphate and no formation of phosphoric esters.

If, however, NaF is added in concentrations as low as $M/200$, although added glycogen is broken down at the same rate, no glucose is formed. Instead, a rapid esterification of inorganic phosphate occurs exactly equivalent to the amount of glycogen disappearing; i.e. one mol. P to one glucose unit of glycogen. The inorganic phosphate which has disappeared all appears as a difficultly hydrolysable phosphoric ester which was identified as hexose 6-phosphate (the Embden equilibrium ester).

If in the presence of NaF more glycogen is added than phosphate, glycogen breakdown goes on until all the inorganic phosphate has been esterified and then ceases, and the excess glycogen remains unaffected. In the absence of NaF, however, glycogen breakdown proceeds at a steady rate for periods up to 2 hr. at least, even with very low concentrations of phosphate.

The only probable explanation of these experiments is the following one. The first step in the breakdown of glycogen in the liver is phosphorolysis, by which it is combined with phosphate to form Cori ester; this reaction is not affected by NaF. In normal brei, the Cori ester is hydrolysed as soon as it is formed, to glucose and phosphate. The net result of these two reactions is that glycogen is quantitatively converted into glucose.



As we shall show later, reaction (2) is considerably faster than reaction (1); this explains why no esterification of inorganic phosphate is found during the breakdown of glycogen in normal brei.

It will be seen that in the above scheme there is a circulation of phosphate between inorganic and organic combination; thus small amounts of phosphate can bring about the breakdown of large amounts of glycogen, playing the part of a "coenzyme" in the conversion of glycogen into glucose in the liver.

In the presence of NaF, on the other hand, the dephosphorylation of Cori ester to glucose is inhibited and under these conditions the Cori ester is converted, not into glucose, but into Embden ester; therefore, in presence of NaF, glycogen is quantitatively converted into hexose 6-phosphate, as it is in normal muscle extract.

By this means, if there is more glycogen present than phosphate the inorganic phosphate will ultimately all be esterified and so no more glycogen can be phosphorylated. This explains the experimental observations mentioned above.

In confirmation of this hypothesis we find that Cori ester added to normal liver brei is very rapidly dephosphorylated; in the presence of NaF, however, the dephosphorylation to glucose is inhibited and little inorganic phosphate liberated; instead, the Cori ester is mainly converted into the Embden ester. This explains why, in presence of NaF, glycogen is converted not into glucose but into Embden ester.

Embden ester is not the only product formed, however, when Cori ester is added to NaF-poisoned liver brei. There is also a synthesis of glycogen, or at least of a polysaccharide indistinguishable from glycogen by all the chemical and physical tests applied. After 15 min. about 25 % of the added Cori ester is converted into glycogen, and the rest into Embden ester. This synthesis of glycogen from Cori ester is a reversal of the process of phosphorolysis of glycogen, and is accompanied by a liberation of inorganic phosphate; thus in liver, as well as in muscle and in yeast, the reaction

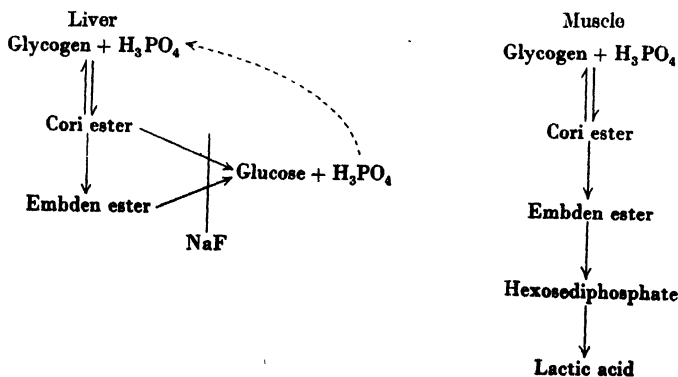


is shown to be reversible.

Thus there are three ways in which the Cori ester may be metabolised in the liver: (a) dephosphorylation to glucose, (b) conversion into Embden ester, (c) synthesis to glycogen. Of these (a) is the most rapid and if Cori ester is added to liver brei it is almost all converted into glucose. This reaction, however, unlike (b) and (c), is inhibited by NaF, and if NaF is present only (b) and (c) can take place. In the absence of NaF, no synthesis of glycogen from Cori ester takes place, since (a) being much faster occurs almost exclusively.

Not only Cori ester is dephosphorylated to glucose by liver tissue; the Embden ester is also dephosphorylated, and since Embden ester accumulates from glycogen in NaF-poisoned liver brei, this dephosphorylation is also presumably inhibited by NaF.

We may therefore draw up the following schemes, comparing the breakdown of glycogen in liver and in muscle:



EXPERIMENTAL

Methods

Preparation of liver brei. Rabbits starved 24–48 hr. were killed by a blow on the neck, and bled at the throat as thoroughly as possible; the livers were then rapidly removed, the gall-bladder excised, and adherent blood washed off.

No further precautions were taken to render the livers free from blood. They were then minced in a Latapie mincer, and weighed amounts of the minced tissue ground in a mortar with an equal volume of fluid containing the necessary substances. The brei was allowed to stand at room temperature, samples being taken at intervals.

Cori ester. The Cori ester used was the di-potassium salt. Some of it was prepared from muscle by the method of Kiessling [1938]; most of it was prepared from pea meal extracts by the method of Hanes [1939]. We wish to express our thanks to Dr Hanes for his great kindness in providing us with large amounts of the pea enzyme, and affording invaluable assistance in the preparation of the Cori ester.

Methods of estimation. Phosphate was estimated in trichloroacetic acid filtrates by the method of Fiske & Subbarow [1925]. Glycogen was estimated in most cases by the method of Good *et al.* [1933]. In some cases it was estimated by precipitation from trichloroacetic acid filtrates by the addition of three volumes of alcohol, and subsequent hydrolysis. Glucose was estimated by the method of Hagedorn & Jensen [1923] in trichloroacetic acid filtrates after neutralization.

(1) *Phosphorylation of glycogen and formation of hexosemonophosphate and glucose*

Table I. *Phosphorylation of glycogen in liver brei, and effect of NaF*

Rabbit starved 24 hr. Dilution of brei 1/1. Room temperature. All figures in mg. per 10 ml. brei.

Exp.	Additions	Time (min.)	Inorganic P	Difficultly hydrolysable P*
1	0.4% glycogen (40 mg.) <i>M</i> /45 phosphate† (6.7 mg. P)	3	8.25	5.0
		30	7.70	5.0
		60	8.40	4.6
		120	9.10	4.2
2	Do. + <i>M</i> /200 NaF	3	7.35	5.85
		30	3.10	8.55
		60	1.90	9.45
		120	1.65	9.55
3	None	0	1.67	5.25

* Calculated from $P_{\text{total}} - P_{30 \text{ min.}}$

† In this and all other experiments, unless stated to the contrary, Sørensen's phosphate buffer, pH 7.2, was used.

If glycogen and phosphate are added to liver brei in the presence of *M*/200 NaF there is a rapid disappearance of inorganic P, and a parallel formation of "difficultly-hydrolysable" organic P (i.e. organic P not hydrolysable in 30 min. by *N* HCl at 100°). As we shall show later, this difficultly-hydrolysable ester, formed by the phosphorolysis of glycogen by inorganic P, is hexosemonophosphate.

In the absence of NaF, however, there is no esterification of inorganic P, and no accumulation of difficultly-hydrolysable organic P—in fact a small decrease, due to hydrolysis of the phosphoric esters of the liver tissue.

In the experiment shown in Fig. 1, the conditions were exactly the same as in Table I, but the disappearance of glycogen was measured, as well as that of inorganic P.

Glycogen breaks down at almost exactly the same rate in the presence or absence of NaF, but in absence of NaF almost no inorganic P is esterified, while in presence of NaF an amount of inorganic P is taken up almost exactly equal to the glycogen broken down.

This clearly shows that NaF has no effect on the phosphorylation of glycogen, but only affects the dephosphorylation of the phosphoric ester formed.

These, and all the following experiments, were done on rabbit liver brei. In a few experiments (not shown) rat and pigeon livers were used; the results were exactly the same as those obtained with rabbit liver.

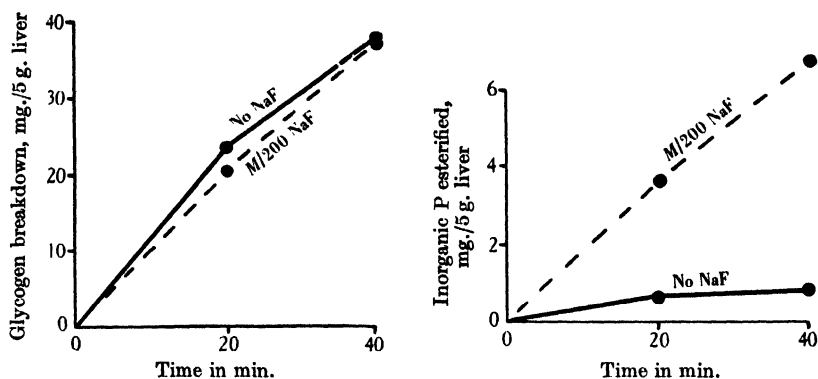


Fig. 1. Breakdown of glycogen and esterification of inorganic phosphate in liver brei with low concentrations of glycogen and phosphate. Rabbit starved 48 hr. Dilution of brei 1/1. Room temperature. Additions: 0.4% glycogen, M/37.5 phosphate, M/200 NaF.

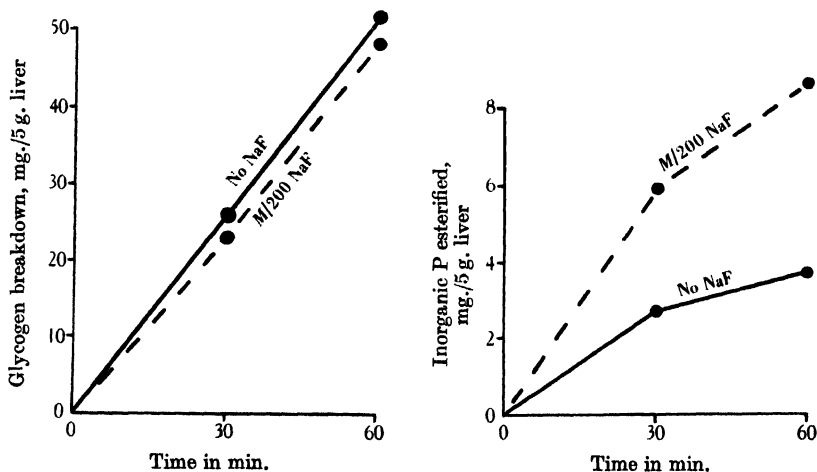


Fig. 2. Breakdown of glycogen and esterification of inorganic phosphate in liver brei with high concentrations of glycogen and phosphate. Rabbit starved 24 hr. Dilution of brei 1/1. Room temperature. Additions: 1.0% glycogen, M/15 phosphate, M/200 NaF.

Although in the above experiments no P is esterified in the absence of NaF, a direct demonstration of the phosphorylation of glycogen in unpoisoned liver is possible if high concentrations of glycogen and phosphate. In the experiment shown in Fig. 2 concentrations of glycogen and phosphate are $2\frac{1}{2}$ times as great as in Fig. 1, and here there is a definite esterification of P even without NaF. The P esterified, however, is only equivalent to about $\frac{1}{3}$ of the glycogen broken down, while in the presence of NaF the P esterified is almost exactly equivalent to the glycogen disappearing.

As we shall show later, the reason for the uptake of P in presence of NaF is that the NaF inhibits the dephosphorylation of the Cori ester formed by the phosphorylation of glycogen; it is probable that high concentrations of phosphate have the same effect to a lesser degree, since it is a well-known fact that phosphatases are inhibited by inorganic phosphate [e.g. Martland & Robison, 1927].

Table II. *Effect of phosphate concentration on glycogen breakdown in liver brei*

Rabbit starved 24 hr. Dilution of brei 1/1. Room temperature. All figures in mg. per 10 ml. brei.

Exp.	Additions	Time (min.)	Glycogen broken down†	Inorganic P esterified†
1	1.5% glycogen*	30	25	0.13
		60	43	0.07
2	Do. + <i>M</i> /45 phosphate	30	35	1.2
		60	50	1.8
3	Do. + <i>M</i> /15 phosphate	30	39	3.4
		60	67	4.8

* In this sample, where no phosphate was added, the initial inorganic P content of the liver tissue was 0.326 mg. per g., i.e. the brei diluted 1/1 contained *ca.* *M*/200 phosphate.

† Amount present after *x* min. subtracted from amount initially present.

In Table II it is shown that addition of phosphate increases the rate of glycogen breakdown in liver brei. The stronger the added phosphate, the faster the glycogen breaks down.

Confirming the results shown in Fig. 2, there is a definite esterification of phosphate (in absence of NaF) in the experiment with *M*/15 phosphate, but a much smaller esterification with *M*/45 phosphate. Even in the former case, the P esterified is much less than the glycogen broken down.

The fact that glycogen breakdown is accelerated by phosphate confirms the theory that phosphorylation is the main mode of glycogen breakdown in the liver.

This is further confirmed by the experiments shown in Table III and Fig. 3, where the breakdown of glycogen and the esterification of phosphate were simultaneously measured, with and without fluoride, the amount of glycogen added being three times higher than the amount of phosphate needed to esterify it. In the experiments with and without NaF, the rates of glycogen breakdown run parallel for the first 30 min.; at this time, when about 1/3 of the added glycogen has vanished, nearly all the inorganic P has disappeared in the experiment with NaF, though in the experiment without NaF none has disappeared. At this point, in the experiment with NaF, where little inorganic phosphate remains, the rate of glycogen breakdown changes abruptly and thereafter proceeds at only 1/5 of its former velocity, while in the experiment without NaF glycogen breaks down at the same rate as before.

This is explained as follows. In absence of NaF, glycogen is phosphorylated, with uptake of inorganic P to form Cori ester. This is then dephosphorylated to glucose, and the esterified phosphate is set free, to be used for the phosphorylation of more glycogen. Thus, even if the phosphate concentration is very low, glycogen can still break down (cf. Table II, exp. 1). In the presence of NaF, however, the Cori ester formed is not dephosphorylated, so the esterified P is not set free again, and, when it has all gone, no more glycogen can be phosphorylated.

Table III. *Glycogen breakdown in liver brei—glycogen concentration three times higher than the equivalent amount of phosphate present*

Rabbit starved 24 hr. Dilution of tissue 4/5. Room temperature. All figures in mg. per 4 g. liver.

Exp.	Additions	Time min.	Glycogen present	Inorganic P present
1	<i>M</i> /67.5 phosphate	3	0	6.07
		30	0	6.67
		90	0	6.36
2	<i>M</i> /67.5 phosphate + 1.1 % glycogen	3	88.3	5.85
		30	68.5	6.07
		90	34.9	6.75
3	Like (2) + <i>M</i> /200 NaF	3	88.3	5.21
		30	75.5	1.47
		90	70.5	1.35

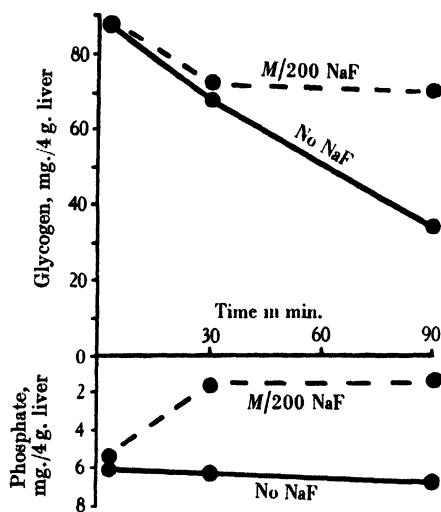


Fig. 3. Glycogen breakdown in liver brei: glycogen concentration three times higher than the equivalent amount of phosphate present. For details see Table III.

The slow breakdown of glycogen that then occurs is probably due, in part at least, to amylase activity. In the period from 30 to 90 min. in the experiment with NaF 5 mg. of glycogen are broken down; in the experiment without NaF 37.6 mg. Therefore only 13.3 % of the observed glycogen breakdown in the liver under these conditions can be due to amylase activity; the rest is due to the phosphorylating mechanism.

(It will be observed that in the experiment with NaF, though the inorganic P sinks to a very low level, it does not entirely disappear. It is not improbable that the inorganic P apparently found arises from the decomposition of labile phosphoric esters on standing in the acid molybdate reagent, since no attempt was made in this work to measure the true inorganic P as distinct from the "directly-estimable" P.)

In Table IV are shown the effects of various poisons on the rate of glycogen disappearance and uptake of inorganic P in liver brei. With NaF, as in previous experiments, glycogen breaks down at an undiminished rate, and a nearly equivalent amount of inorganic P is esterified. With iodoacetate, however, no

Table IV. *Effect of various poisons on the phosphorylation of glycogen in liver brei*

Mixed liver tissue of two rabbits, starved 24 hr. Dilution of brei 1/1. Incubated at room temperature. All figures in mg./10 ml. brei. All experiments contained *M*/7.5 phosphate.

Exp.	Additions	Time (min.)	Inorganic P esterified	Glycogen broken down
1	<i>M</i> /200 NaF	45	3.5	—
		120	2.0	—
2	2% glycogen + <i>M</i> /200 NaF	45	11.0	77
		120	17.7	140
3	2% glycogen + <i>M</i> /400 iodoacetate	45	2.9	45
		120	4.9	124
4	2% glycogen + <i>M</i> /100 phloridzin	45	1.0	26
		120	0.7	54

more phosphate is taken up than in the control without glycogen, though the breakdown of glycogen is little affected. (The small inhibition observed—11% after 1 hr.—may be attributed to the general toxic effect of iodoacetate in the concentration used.) This shows that oxido-reductive processes are not concerned in the phosphorylation of glycogen.

With phloridzin also, esterification of phosphate is no larger than in the control, but here the breakdown of glycogen is greatly inhibited—nearly 70%. This is of the same order as the inhibition of phosphorolysis by phloridzin in muscle extract or brei, and strongly supports the view that glycogen breakdown in liver is mainly brought about by phosphorylation.

On the same sample of liver used in this experiment, attempts were made to demonstrate a phosphorylation of glucose and fructose. The results (not shown) were completely negative; no phosphorylation could be detected, aerobically or anaerobically, even in presence of NaF.

This of course does not prove that glucose and fructose cannot under any circumstances be phosphorylated in the liver. It does show, however, that under conditions where glycogen is rapidly phosphorylated glucose and fructose are not, and therefore neither glucose nor fructose can be intermediates in the breakdown of glycogen; as in muscle, it is the glycogen molecule itself that is phosphorylated.

Table V. *Phosphorylation of glycogen and formation of hexosemonophosphate under aerobic and anaerobic conditions*

Same sample of liver brei as in Table IV. Dilution of brei 1/1. Diluted brei shaken in Krebs bottles in a thermostat at 37°, in atmospheres of pure N₂ or O₂, for 35 min. Hexosemonophosphate isolated as described in text. All figures in mg./10 ml. brei.

Exp.	Additions	Gas	Inorganic P esterified	Hexose-monophosphate formed as mg. P
1	<i>M</i> /7.5 phosphate + <i>M</i> /200 NaF	O ₂	0	Trace
2	Do.	N ₂	— 2.8	Trace
3	Do. + 2% glycogen	O ₂	17.1	10.1
4	Do. + 2% glycogen	N ₂	11.6	8.16

In the experiments shown in Table V, the phosphorylation of glycogen under aerobic and anaerobic conditions in the presence of NaF was measured, and the phosphoric ester which accumulates as a result of this phosphorylation was isolated in the following way.

After deproteinization with trichloroacetic acid, the filtrate was made

alkaline to phenolphthalein with strong NaOH, and excess 50 % Ba acetate added. After standing, the precipitate was centrifuged off and rejected. The supernatant fluid was treated with 2 vol. of alcohol and kept in the refrigerator overnight. The precipitate was centrifuged off, washed with alcohol, dissolved in water, the small insoluble residue centrifuged off and the supernatant again precipitated with 2 vol. EtOH after adding a few drops of $N/10$ $Ba(OH)_2$. The precipitated Ba salt was washed with alcohol and ether and dried in a desiccator. The salts isolated from exps. 3 and 4, Table V, contained respectively 7 and 7.2 % of P (of which no more than a trace was inorganic P), and had ratios of $\frac{\text{Reduction (Hagedorn-Jensen)}}{\text{Organic P}}$ of 3.67 and 3.87. The theoretical values for pure Embden ester are 7.86 % P, and $\frac{\text{Reduction}}{\text{P}} = 3.88$ %. The values for the isolated salts are thus a little low, but it is very difficult to obtain the Ba salts completely dry. From the analytical figures, the method of isolation and the hydrolysis curves in N HCl, which agreed with that of the Embden ester, there can be little doubt that the Ba salts isolated were those of the Embden hexosemonophosphate.

In the aerobic experiment 60 % of the inorganic P esterified was isolated as hexosemonophosphate; in the anaerobic experiment 70 %. Allowing for the losses in isolation, which are considerable (20–25 %), it seems probable that, in NaF-poisoned brei, the glycogen phosphorylated is quantitatively converted into Embden ester, at any rate under anaerobic conditions.

The rate of phosphorylation of glycogen is faster in O_2 than in N_2 . We suggest that this is because hexosemonophosphate accumulates anaerobically, while it is removed aerobically by direct oxidation [cf. Warburg & Christian, 1937; Lipmann, 1936; Dickens, 1936; 1938]. This would account for the lower percentage yield and slightly lower purity of the hexosemonophosphate isolated in the aerobic experiment.

The speed of phosphorolysis was very great in this experiment. At 37° and with a glycogen concentration of 2 %, 5 g. liver can bring about the disappearance of 17.1 mg. P in 35 min., corresponding to the phosphorylation of 3.06 g. glycogen per 100 g. liver per hr. At room temperature the velocity is less than half of this. It also depends on the glycogen concentration, and falls to a value of less than 1 g. per 100 g. liver per hr. if the glycogen concentration is less than 0.5 %, and, as shown in Table II, it also depends on the phosphate concentration. In addition there is considerable variation from one liver to another.

In another experiment glycogen breakdown was measured directly, on the same liver, at 18° and at 37° , using 1.5 % glycogen and $M/15$ phosphate. (For the experiment at 37° , the brei was shaken in a Krebs bottle in a thermostat in an atmosphere of N_2 .) The rate of glycogen breakdown at 18° was 1.04 g. per 100 g. liver per hr.—rather less than usually found. The rate at 37° was very high indeed—4.06 g. per 100 g. liver per hr.

The experiments shown in Table VI illustrate the “sugar” formation from glycogen in presence and absence of NaF. In both cases there is a rapid increase in reducing value; in the brei containing NaF, however, this only reaches 2/3 of the value reached in the “plain” sample. In the sample with NaF we find, as usual, a rapid esterification of inorganic P, while none occurs without NaF.

If we assume that in the experiment with NaF all the P esterified has been converted into the Embden ester, the calculated reduction values for the 30, 60 and 150 min. periods (obtained by multiplying the amounts of phosphate esterified by 3.94; i.e. the ratio $\frac{\text{Reduction}}{\text{P}}$ of the Embden ester) are 23.6, 25.6 and 26.4. The observed values, corrected for the control, are 24.1, 26.4 and 26.6.

Table VI. *Breakdown of glycogen and glucose formation in liver brei in presence and absence of NaF*

Rabbit starved 24 hr. Dilution of brei 1/1. Room temperature. Reduction determined in trichloroacetic acid filtrates (after neutralizing) by method of Hagedorn & Jensen. All figures in mg./10 ml. brei.

Exp.	Additions	Time (min.)	Inorganic P esterified	Increase in reduction as mg. glucose
1	None	30	- 0.04	1.6
		60	- 1.0	1.8
		150	- 1.6	2.7
2	0.5% glycogen M/45 phosphate	30	- 0.05	38.7
		60	- 0.45	40.5
		150	- 1.85	43.2
3	0.5% glycogen M/45 phosphate M/200 NaF	30	6.0	25.7
		60	6.5	28.2
		150	6.7	29.2

Recovery of added glucose

5 g. liver + 5 ml. H ₂ O	Glucose found, 29.3 mg.
5 g. liver + 5 ml. 1% glucose (50 mg.)	Glucose found, 78.4 mg.

Recovery = 98.4%.

If we assume that in the experiment without NaF the same breakdown of glycogen has occurred, but that the product is glucose instead of Embden ester, the amount of glucose formed in exp. 2 can be calculated from the amount of P esterified in exp. 3. The calculated values for the 30, 60 and 150 min. periods are 34.8, 37.8 and 38.9; the observed values (corrected for the control exp.) are 37.1, 38.9 and 40.5.

We conclude that, with or without NaF, the same amounts of glycogen undergo phosphorylation to Cori ester; without NaF the Cori ester is all hydrolysed to glucose, while with NaF this reaction is inhibited and it is all converted into Embden ester. The good agreement between observed and calculated reduction values supports this theory.

Glucose added to the brei was quantitatively recovered (98.4%) and, up to 150 min., did not disappear. This shows (a) that the technique used was reliable, and (b) that glucose is not metabolized by the liver to any significant extent under these conditions, which are virtually anaerobic.

(2) *Dephosphorylation of Cori ester and glycogen synthesis*

Cori ester added to liver brei is very rapidly dephosphorylated, as shown in Fig. 4. The rate depends on the concentration of Cori ester; with high concentrations it is very high indeed. The initial velocity in curve 1, Fig. 4, corresponds to a hydrolysis of 3.9 g. Cori ester (expressed as glucose formed) per 100 g. liver per hr. at room temperature.

It has already been suggested that the dephosphorylation of Cori ester is inhibited to some extent by high concentrations of phosphate. Table VII shows that glucose also causes partial inhibition. This is similar to the hydrolysis of glycerophosphate by bone phosphatase, which is inhibited by both glycerol and phosphate [Martland & Robison, 1927].

Table VIII shows that the dephosphorylation of the Cori ester is inhibited by NaF. This affords direct proof of our theory that, in normal liver brei, glycogen

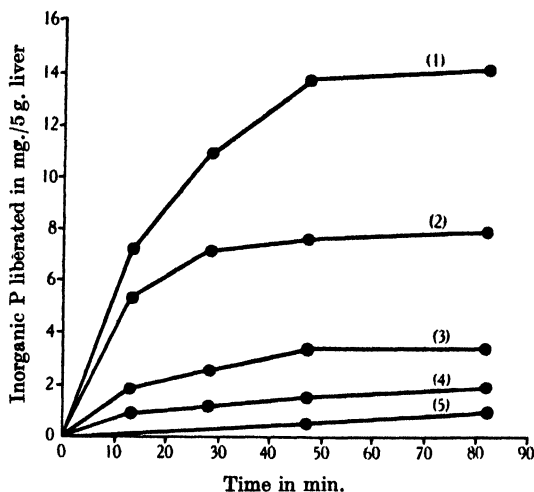


Fig. 4. Dephosphorylation of Cori ester in liver brei. Rabbit starved 48 hr. Dilution of brei 1/1. Room temperature. Curve 1: Cori ester, 2.0 mg. P per ml.; Curve 2: Cori ester, 1.0 mg. P per ml.; Curve 3: Cori ester, 0.33 mg. P per ml.; Curve 4: Cori ester, 0.13 mg. P per ml.; Curve 5: no additions.

Table VII. *Effect of glucose on dephosphorylation of Cori ester on liver brei*

Rabbit starved 24 hr. Dilution of brei 1/1. Room temperature. All figures in mg./10 ml. brei.

Exp.	Additions	Time min.	Inorganic P formed
1	None	15	0.30
		30	0.37
2	Cori ester (12.5 mg. P)	15	5.77
		30	7.72
3	Do. + 2% glucose	15	3.83
		30	5.45

Table VIII. *Effect of NaF on dephosphorylation of Cori ester in liver brei*

Rabbit starved 36 hr. Dilution of brei 1/1. Room temperature. Glycogen measured in initial and 15 min. periods only. All figures in mg./10 ml. brei.

Exp.	Additions	Time min.	Inorganic P formed	Glycogen formed
1	None	15	0.4	0
		30	0.65	—
		60	1.3	—
2	Cori ester (5.5 mg. P)	15	3.15	0
		30	4.00	—
		60	4.55	—
3	Cori ester (5.5 mg. P) + $M/200$ NaF	15	0.57	7.6
		30	-0.85	—
		60	-0.85	—

is phosphorylated to Cori ester, which is then hydrolysed to glucose and phosphate so fast that there is no observable uptake of phosphate and very little formation of Embden ester; with NaF present the Cori ester cannot be dephosphorylated to glucose, and is therefore converted into Embden ester. The result

is that phosphate is taken up and Embden ester formed in amounts equivalent to the glycogen broken down and there is no formation of glucose.

When glycogen is phosphorylated, the Cori ester produced is converted as fast as it is formed into either Embden ester or glucose, and never accumulates to any extent. (We have never observed any considerable accumulation of easily hydrolysable P during the phosphorylation of glycogen, with or without NaF.) But when Cori ester itself is added in high concentration to NaF-poisoned brei in the absence of glycogen and phosphate, a new phenomenon makes its appearance—the synthesis of glycogen. This does not happen without NaF, for then the Cori ester is converted into glucose so rapidly that there is no time for any glycogen to be formed. But when this pathway of escape is blocked by NaF, Cori ester remains in fairly high concentration in the liver for a relatively long time, and during this time some of it is converted into glycogen.

In this experiment, in the presence of NaF, about 25% of the added Cori ester was converted into glycogen in the first 15 min.; without NaF no glycogen was formed.

Table IX. *Synthesis of glycogen from Cori ester in NaF-poisoned liver brei*

Rabbit starved 36 hr. Dilution of brei 1/1. Room temperature. All figures in mg./10 ml. brei.

Exp.	Additions	Time min.	Inorganic P formed	Glycogen formed
1	<i>M</i> /200 NaF	3	0	0
		15	–0.15	0
		30	–0.30	0
		60	–0.25	0
2	Cori ester (15 mg. P) + <i>M</i> /200 NaF	3	1.75	4.7
		15	3.45	10.0
		30	3.05	4.8
		60	2.75	0.5

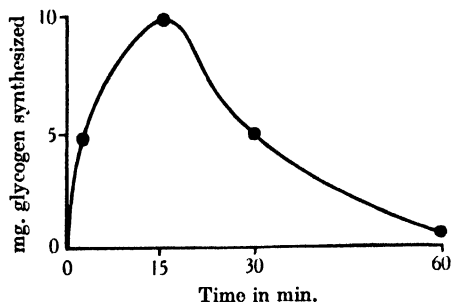


Fig. 5. Synthesis of glycogen from Cori ester in NaF-poisoned liver brei. For details see Table IX.

In Table IX and Fig. 5 the synthesis of glycogen from Cori ester was followed over time intervals from 3 to 60 min. In the presence of NaF glycogen is rapidly synthesized during the first 15 min. and then disappears again, falling almost to zero in 1 hr.¹ This is because, of the two reactions Cori ester can undergo in NaF-poisoned brei,

- (1) Cori ester \rightleftharpoons glycogen + phosphate,
- (2) Cori ester \rightarrow Embden ester,

only the first is reversible. The equilibrium between Cori ester and glycogen is therefore continually disturbed by the irreversible transformation of Cori ester

¹ A similar effect was observed in muscle by Cori *et al.* [1939, 3].

into Embden ester, which ultimately goes to completion. Without NaF, or without Cori ester, no glycogen is formed at any stage; in the former case, the Cori ester is all converted into glucose.

In the experiment with NaF there is a liberation of phosphate parallel to the formation of glycogen, which disappears again as the glycogen disappears. This inorganic phosphate does not come from the dephosphorylation of Cori ester to glucose, but from its synthesis to glycogen and phosphate.

The properties of the glycogen synthesized from Cori ester (isolated by alcohol precipitation from trichloroacetic acid filtrates, and purified by reprecipitation from aqueous solution) are as follows:

(1) It is a white amorphous powder, forming a strongly opalescent solution in water.

(2) The aqueous solution gives with I_2 a red-brown colour of the same shade and intensity as a solution of authentic glycogen of the same strength.

(3) The substance is non-reducing, but liberates the theoretical amount of glucose after 2 hr. hydrolysis at 100° in 0.6 *N* HCl. It is not affected by 30 % KOH.

(4) A solution containing 0.98 mg. per ml. gave a rotation in a 2 dm. tube of 0.37° . $[\alpha]_D + 188^\circ$.

The substance is therefore indistinguishable from glycogen.

Table X. *Formation of Embden ester and glycogen from Cori ester in NaF-poisoned liver brei*

Rabbit starved 36 hr. Dilution of brei 1/1. Room temperature. Incubation time 15 min. in both experiments. All figures in mg./10 ml. brei.

Exp.	Additions	Embden ester found (as mg. P)	Glycogen found
1	<i>M</i> /200 NaF	1.42	0
2	Cori ester (18.75 mg. P) + <i>M</i> /200 NaF	14.06	12.8

In Table X Cori ester was added to NaF-poisoned liver brei, and Embden ester and glycogen were isolated as previously described. In the control without Cori ester no glycogen and almost no Embden ester were found. With Cori ester, however, there was a large and rapid formation of both these substances. The Ba salt isolated had a ratio of $\frac{\text{Reduction}}{\text{Organic P}}$ of 3.85, and its hydrolysis curve in *N* HCl agreed with that of the Embden ester, so there is no doubt of its identity.

Allowing for the small loss in isolation of the Embden ester, and the rather larger loss incurred in the purification of the glycogen, it will be seen that in the 15 min. of the experiment Cori ester is converted into Embden ester and glycogen in the approximate ratio of 3/1.

In the experiments shown in Table XI, the enzyme dephosphorylating Cori ester was investigated in rather more detail. The possibility was considered that not only an enzyme, but also a coenzyme might be involved; for example, phosphate might be transferred from Cori ester to adenylic acid, forming glucose and adenylypyrophosphate, the latter then being dephosphorylated. The addition of adenylic acid to the brei, however, caused no increase in the rate of dephosphorylation of Cori ester.

It was also found that Embden ester is dephosphorylated by liver brei at about the same rate as Cori ester; here again the addition of adenylic acid caused no increase in the rate of dephosphorylation.

Table XI. *Dephosphorylation of Cori ester and Embden ester in liver brei and the effect of adenylic acid*

Rabbit starved 48 hr. Dilution of brei 1/1. Room temperature. All figures in mg./5 g. liver.

Exp.	Additions	Time min.	Inorganic P
1	None	2	1.60
		15	1.72
		30	1.85
		60	2.12
2	Adenylic acid* (1.79 mg. P)	2	1.82
		15	1.72
		30	1.96
		60	2.28
3	Cori ester (4.5 mg. P)	2	2.72
		15	4.43
		30	4.88
		60	5.10
4	Cori ester (4.5 mg. P) + adenylic acid (1.79 mg. P)	2	2.76
		15	4.43
		30	5.10
		60	5.57
5	Embden ester (5 mg. P)	2	2.00
		15	4.90
		30	5.20
		60	5.55
6	Embden ester (5 mg. P) + adenylic acid (1.79 mg. P)	2	1.93
		15	4.43
		30	5.00
		60	5.55

* Muscle adenylic acid.

Table XII. *Dephosphorylation of Cori ester in liver extracts, and the effect of adenylic acid*

Liver of starved rabbit extracted with one part distilled water. Autolysed 5 hr. at room temperature and dialysed 12 hr. against distilled water at 5°. Each experiment contained 3 ml. extract made up to 5 ml. with additions. All figures calculated for whole sample. Extracts incubated at room temperature.

Exp.	Additions	Time min.	Inorganic P formed mg.
1	None	15	0.03
		30	0.07
		60	0.15
2	Adenylic acid (0.88 mg. P)	15	0.08
		30	0.13
		60	0.36
3	Cori ester (1.5 mg. P)	15	0.72
		30	1.11
		60	1.53
4	Cori ester (1.5 mg. P) + adenylic acid (0.88 mg. P)	15	0.86
		30	1.36
		60	1.73

The above experiments do not entirely exclude the possibility that adenylic acid may be concerned in the dephosphorylation of Cori ester, since it might be that there was already enough adenylic acid in the liver to "saturate" the enzyme. Experiments were therefore made (Table XII) on the effect of adenylic acid on the dephosphorylation of Cori ester in liver extracts autolysed 5 hr. to destroy

adenylic deaminase and dialysed to remove adenylic acid. Here again adenylic acid does not accelerate the dephosphorylation, which seems to show that adenylic acid does not act as a phosphate-carrier in this reaction.

Table XIII. *Dephosphorylation of Cori ester in liver extracts. Effects of dialysis, Mg and NaF*

Liver of 48 hr.-starved rabbit extracted with 1 vol. distilled water at 0°. Dialysed for 14 hr. against running distilled water at 5°. In each exp. 2 ml. extract made up to 4 ml. with additions. Incubated at room temperature. Figures in mg. calculated for whole sample.

Exp.	Additions	Time min.	Inorganic P formed
1	None	30	0.04
		60	0.10
2	Cori ester (4.24 mg. P)	30	2.02
		60	3.40
3	Do.	30	1.94
		60	3.62
4	Do. + MgCl ₂ (4 mg. Mg)	30	1.84
		60	3.33
5	Do. + M/200 NaF	30	0.80
		60	1.24

Exps. 1, 2, 4 and 5. Dialysed extract. Exp. 3. Undialysed extract.

Table XIII shows further experiments on liver extracts. The velocity of dephosphorylation of Cori ester is the same in dialysed and undialysed extracts. This seems to show that no coenzyme is required; however, we should not like to commit ourselves on this point, since it is possible that a coenzyme is involved which is difficult to remove by dialysis. (The difficulty of completely removing cozymase from muscle extracts, even by prolonged dialysis, is well known.)

Mg, however, is fairly easily removed by dialysis, and since dialysis causes no inhibition, and addition of Mg to the dialysed extract causes no acceleration, we may conclude that the enzyme dephosphorylating the Cori ester, unlike some phosphatases, does not require Mg.

Table XIV. *Dephosphorylation of hexosediphosphate by liver brei*

Rabbit starved 24 hr. Dilution of liver brei 1/1. Room temperature. All figures in mg./10 ml. brei.

Exp.	Additions	Time min.	Inorganic P formed
1	None	15	0.17
		30	0.21
		60	0.87
2	Hexosediphosphate (10.5 mg. P)	15	0.54
		30	0.86
		60	1.46
3	Do. + M/200 NaF	15	0.04
		30	0.04
		60	0.17

The specificity of the enzyme hydrolysing the Cori ester has not been thoroughly investigated. Tables XI and XII show that Cori ester and Embden ester are hydrolysed at about the same rate, while adenylic acid is only very slowly dephosphorylated. Table XIV shows that hexosediphosphate is only very slowly hydrolysed.

As far as is known, therefore, the enzyme only hydrolyses the 1- and 6-hexose-monophosphates.

(3) *Synthesis of glycogen from glucose in liver slices*

In none of the experiments we have carried out on liver brei have we succeeded in demonstrating either the synthesis of glycogen from glucose or fructose, or even the phosphorylation of these sugars. In the living animal, on the other hand, both these substances, and many others, give rise to liver glycogen, and it has been possible to demonstrate glycogen formation *in vitro* by liver slices in their presence [Cross & Holmes, 1937]. Experiments with liver slices have therefore been undertaken in connexion with the present work.

The experiments of Cross & Holmes were all made by shaking the liver slices in a NaHCO_3 -buffered medium containing sugar in an atmosphere of $\text{O}_2\text{-CO}_2$. Since glycogen is formed in liver from glucose 1-phosphate, it seemed likely that glycogen synthesis in the slices should go forward, and might be increased, in a phosphate buffer, though of course it was possible that no phosphate would penetrate the cells. Since the synthesis of glycogen from Cori ester in brei can only be demonstrated in the presence of NaF , it seemed necessary to test the effect of NaF on liver slices. Ca is precipitated by phosphate buffer of the strength and pH used in the experiments with brei, so that it was omitted from the Ringer solution used for the liver slices shaken in phosphate buffer; the fluid used contained Na , K and Mg in the proportions used by Krebs [1933], and various concentrations of phosphate. The pH was 7.2. The glycogen determinations were carried out by adding the required amount of 80% KOH to the tissue while it was still being shaken in the thermostat as previously described [Cross & Holmes, 1937]. We investigated the synthesis of glycogen both after long and short periods of incubation; we also tested the effect of aerobic and anaerobic conditions on the synthesis, and the effect of insulin.

Our findings may be summarized as follows. Liver slices synthesize glycogen from glucose in Krebs Ringer solution containing Ca and buffered with CO_2 and NaHCO_3 . The synthesis only occurs under aerobic conditions; no synthesis, or even a slight decrease in glycogen is observed anaerobically. The synthesis is not increased, but slightly decreased by insulin. It is relatively slow, the highest values being obtained after $2\frac{1}{2}$ hr., the longest time for which experiments were continued. If the fluid is buffered by phosphate instead of NaHCO_3 , the synthesis is very small. This is partly due to the absence of Ca , for if the phosphate concentration is lowered to $M/60$, so that Ca can be added to the Ringer solution, the synthesis is somewhat increased, and approaches the value found in NaHCO_3 -buffered but Ca -free Ringer solution. If Ca is added to the NaHCO_3 -Ringer solution in various concentrations, the maximum synthesis is observed when the concentration of Ca is 10 mg./100 ml.; i.e. approximately that found in blood. NaF completely inhibits the synthesis, whether the Ringer solution be buffered with phosphate or NaHCO_3 .

Similar experiments were done with brei, but no synthesis of glycogen from glucose was ever observed with or without NaF . No accumulation of organic phosphate was observed in the liver slices in any of the experimental conditions investigated either with or without NaF .

These findings are illustrated by the experiments shown in Table XV.

Group A illustrates the synthesis of glycogen in NaHCO_3 -Ringer solution containing glucose. There is no synthesis of glycogen in the absence of glucose.

Group B shows that synthesis of glycogen does not occur anaerobically.

Group C shows that glycogen synthesis is very small in a NaHCO_3 buffer without Ca .

Table XV

Rabbits starved 24 hr. 0.5 g. liver slices used for each experiment, shaken in 3 ml. Ringer solution containing 1% glucose, and otherwise of composition as indicated in col. 6. Ca of usual concentration (10 mg./100 ml.) unless otherwise indicated.

	Glycogen, mg./g. fresh tissue			Time of incubation min.	Gas phase	Composition of fluid
	Initial	Final	Increase			
A	1.36	5.22	3.86	60	O ₂ /CO ₂	NaHCO ₃ /CO ₂ + Ca
	0.74	4.00	3.26	120	"	"
	0.74	4.35	3.61	150	"	"
	0.74	0.53	-0.21	120	"	No glucose
	0.74	0.47	-0.27	150	"	No glucose
	0.15	3.80	3.65	160	"	"
B	1.31	4.93	3.62	120	O ₂ /CO ₂	NaHCO ₃ /CO ₂ + Ca
	1.31	4.95	3.64	120	"	"
	0.72	0.60	-0.08	120	N ₂ /CO ₂	"
	0.60	0.59	-0.01	120	"	"
C	0.31	0.41	0.10	15	O ₂ /CO ₂	NaHCO ₃ /CO ₂ . No Ca
	0.31	0.45	0.14	30	"	"
	0.31	0.36	0.05	60	"	"
	0.31	0.87	0.56	90	"	"
	0.16	0.80	0.64	60	"	"
	0.16	1.10	0.94	120	"	"
	0.15	0.88	0.73	160	"	"
D	0.55	0.35	-0.20	60	O ₂	Phosphate, M/20. No Ca
	0.77	0.32	-0.45	60	"	"
	0.16	0.20	0.04	60	"	Phosphate, M/30. No Ca
	0.16	0.68	0.52	120	"	"
	0.15	0.29	0.14	160	"	Phosphate, M/60. No Ca
	0.15	1.94	1.79	160	"	Phosphate, M/60 + Ca
E	0.36	3.08	2.72	120	O ₂ /CO ₂	NaHCO ₃ /CO ₂ . No Ca
	0.36	7.31	6.95	120	"	NaHCO ₃ /CO ₂ . Ca 5 mg./100 ml.
	0.36	8.76	8.40	120	"	NaHCO ₃ /CO ₂ . Ca 10 mg./100 ml.
	0.36	8.51	8.15	120	"	NaHCO ₃ /CO ₂ . Ca 15 mg./100 ml.
	0.15	0.12	-0.03	160	"	NaHCO ₃ /CO ₂ . No Ca + M/200 NaF
F	0.29	6.16	5.87	120	O ₂ /CO ₂	NaHCO ₃ /CO ₂ . Ca
	0.29	5.33	5.04	120	"	NaHCO ₃ /CO ₂ . Ca + 1 mg. insulin
	0.52	4.27	3.75	120	"	NaHCO ₃ /CO ₂ . Ca
	0.52	3.13	2.61	120	"	NaHCO ₃ /CO ₂ . Ca + 1 mg. insulin

Group D shows that glycogen synthesis is small or absent in a phosphate buffer without Ca, but that appreciable synthesis occurs if the phosphate concentration is decreased and Ca added.

Group E shows the influence of varying concentrations of Ca, and includes an experiment showing the inhibition of glycogen synthesis by NaF.

Group F shows the effect of insulin.

The interpretation of these experiments is difficult. Liver brei and muscle extract will form glycogen from Cori ester, but apparently neither phosphorylate glucose nor form glycogen from it under the conditions which we and other workers have used. On the other hand it is certain that in the living animal both liver and muscle form glycogen from glucose, and we have repeatedly demonstrated that isolated liver slices do so. To say that the special activities of the intact cell are necessary for the process may be true but it is not helpful. It is possible that phosphate fails to penetrate the cell, and it will, if present in any considerable concentration, precipitate Ca at the cell surface and in the fluid. If, in the absence of Ca, glucose cannot penetrate the cell, the removal of Ca may account for the absence of synthesis in phosphate-Ringer solution. Synthesis can

go on in weak phosphate if Ca is added, as it does in a "Ca-free" NaHCO_3 -Ringer solution, but in such a case "Ca-free" is only a relative term, since some Ca must be added with the tissue. The effect of Ca recalls the work of Minot *et al.* [1934], who observed the marked curative effect of Ca on animals whose livers had been damaged by chloroform, carbon tetrachloride and guanidine.

Since NaF inhibits the synthesis in liver slices, it is difficult to obtain any evidence as to whether the glucose is phosphorylated or not, since even in brei the formation of phosphate esters from glycogen can be demonstrated only with difficulty unless NaF is added to prevent subsequent dephosphorylation.

DISCUSSION

We consider that the evidence given lends ample support to the scheme of glycogen breakdown put forward in the introduction, and that phosphorylation is the main path of glycogen metabolism in the liver. We should like to draw particular attention to the high velocities of the reactions involved. 100 g. rabbit liver can phosphorylate 4 g. glycogen per hr. at 37° anaerobically. If we assume that the rate in human liver is about the same, then the average human liver (i.e. of about 1.5 kg.) will phosphorylate about 60 g. glycogen per hr. The rate of dephosphorylation of the Cori ester to glucose is even faster. In the intact liver the velocities must be greater still, since our conditions must have been far from optimum. The velocities of the reactions glycogen \rightarrow Cori ester \rightarrow glucose are therefore more than fast enough to maintain the blood sugar level, even in exercise.

One point in our scheme needs amplification. We have shown that both Cori ester and Embden ester are dephosphorylated in the liver at about the same rates, and also that Cori ester can be converted into Embden ester. During the breakdown of glycogen in normal liver, is the Cori ester itself dephosphorylated, or is it first converted into Embden ester and the Embden ester dephosphorylated? We should not like to be too dogmatic on this point, but it seems probable that the Cori ester formed from glycogen is dephosphorylated immediately, without going to Embden ester. The final result of course would be the same in either case; the glycogen is ultimately all converted into glucose.

If the Cori ester before dephosphorylation were converted into the Embden ester, which is an equilibrium mixture of glucose and fructose 6-phosphates, one would expect that dephosphorylation of the latter would give rise to a mixture of glucose and fructose; however, we could never detect the formation of any fructose during the breakdown of glycogen in normal liver brei. This argument is of course not completely conclusive, since it may be that only the glucose component of the Embden ester is dephosphorylated.

In our experiments, in normal brei, glycogen is entirely converted into glucose, while in NaF-poisoned brei, it all forms Embden ester. Probably both these routes are physiological, and the Embden ester serves as a substrate for the metabolism of the liver itself.

The metabolism of Embden ester in liver and in muscle appears to be different. In muscle, it reacts with adenylypyrophosphate, forming hexosediphosphate and adenylic acid [Ostern *et al.* 1936], but in NaF-poisoned liver brei glycogen is converted almost quantitatively into hexosemonophosphate, which does not seem to react further, at least, under anaerobic conditions. This is doubtless due to the fact that liver contains very little adenylypyrophosphate, and as is well known forms little lactic acid. It seems probable that the Embden ester is not converted into lactic acid to any great extent, but is mainly directly oxidized as it is in yeast and in brain [Lipmann, 1936; Dickens, 1936; 1938; Warburg & Christian, 1937].

From this comparison of the metabolism of glycogen in muscle and in liver we venture to draw some conclusions as to the well-known difference in the effect of adrenaline on these tissues. In liver adrenaline accelerates the conversion of glycogen into glucose; in muscle the formation of lactic acid. Both effects can be attributed to the acceleration of phosphorolysis; they differ in subsequent reactions only. In liver, Cori ester is hydrolysed to glucose; in muscle it is converted into lactic acid, by way of Embden ester and hexosediphosphate. If the action of adrenaline is to accelerate phosphorolysis of glycogen, this would also explain the results of Cori & Cori [1931], who found that injection of adrenaline caused an increase in the hexosemonophosphate content, as well as a decrease in the glycogen content, of intact muscles *in situ*.

It must be pointed out, however, that Cori *et al.* [1939, 1] and Gill & Lehmann [1939] failed to find any effect of adrenaline on phosphorylase activity in muscle extracts, a result we can confirm in liver brei. If adrenaline stimulates phosphorolysis of glycogen, its effect must therefore be an indirect one, possibly depending on the integrity of the living cell.

We have dealt for the most part with the breakdown of glycogen to glucose; there remains to be considered the reverse process. Glycogen is synthesized from glucose in the intact animal, the perfused liver and in liver slices. We have directly shown the synthesis of glycogen from Cori ester in liver brei, and there is no doubt that the enzymic conversion of glycogen into Cori ester is reversible. It therefore seems highly probable that in the synthesis of glycogen from glucose, the glucose is first converted into Cori ester. The mechanism of this process is as yet entirely unknown. We are inclined to doubt that it is a simple reversal of the dephosphorylation of the Cori ester to glucose, for the following reasons: (1) In liver slices, glycogen is only synthesized from glucose aerobically. This suggests that the phosphorylation of glucose is coupled with the energy of some oxidative process, as in yeast, for if it were a simple reversal of the dephosphorylation of Cori ester, it should occur equally readily aerobically and anaerobically. (2) Although it has been stated that the action of phosphatases is reversible, and Martland & Robison [1927] have claimed that glucosemonophosphate and glycerophosphate are synthesized by the action of bone phosphatase on glucose and glycerol respectively in the presence of phosphate, the evidence for this is hardly satisfactory, on account of the small extent of the synthesis, the slowness with which it proceeded, and the enormous concentrations used. In Martland & Robison's experiments, for example, using 50 % glycerol and $M/2$ phosphate, only 5.4 % synthesis occurred after 14 days; using 60 % glucose and $M/8$ phosphate, they obtained a 5.3 % synthesis after 21 days. It must be concluded that phosphatases are reversible to a very small extent, or not at all. In any event, we are not altogether certain that the dephosphorylation of the Cori ester is brought about by a simple phosphatase of the ordinary type. (3) The fact that, in muscle, glucose can be synthesized to glycogen but Cori ester cannot be hydrolysed to glucose, shows that the two processes are not necessarily interdependent.

In yeast, Lutwak-Mann & Mann [1935] have shown that glucose is phosphorylated by adenylypyrophosphate. This is probably not the mechanism in liver, which contains very little adenylypyrophosphate. Finally, the possibility that glucose is phosphorylated first to Embden ester, which is then converted into Cori ester and then into glycogen, can be excluded, since we agree with all other workers in finding that the reaction Cori ester \rightarrow Embden ester is not reversible, and have never observed any synthesis of glycogen from Embden ester.

SUMMARY

1. The breakdown of glycogen has been studied in rabbit liver brei.
2. The main pathway of glycogen breakdown in the liver is through phosphorylation with inorganic phosphate, the product being glucose 1-phosphate (Cori ester), which is then dephosphorylated to glucose. Only a small part (not more than 15 %) of the glycogen breakdown in liver is due to amylase activity.
3. Reasons are given for the supposition that the above scheme represents the mechanism by which liver glycogen is converted into blood sugar *in vivo*.
4. In the presence of NaF, the dephosphorylation of the Cori ester is inhibited, and under these conditions, the Cori ester formed by the phosphorylation of glycogen is all converted into Embden ester.
5. When Cori ester is added to NaF-poisoned liver brei, part of it is converted into Embden ester, and the rest to glycogen. Thus the reaction $\text{glycogen} + \text{phosphate} \rightleftharpoons \text{Cori ester}$ has been shown to be reversible, and it is suggested that the reverse reaction is an intermediate step in the synthesis of liver glycogen from glucose.
6. Neither glucose nor fructose is phosphorylated or converted into glycogen in liver brei. Glycogen is, however, synthesized from glucose in liver slices. This synthesis only occurs aerobically. It is strongly inhibited by NaF, and slightly inhibited by insulin. It does not occur unless Ca is present in the Ringer solution in which the liver slices are suspended.

APPENDIX

Since this work was completed, a paper has appeared by Cori *et al.* [1939, 2] who have arrived at substantially the same conclusions as ourselves. These authors were successful in preparing active liver extracts, and by adsorption on alumina C_γ, elution and ammonium sulphate precipitation, were able to separate the enzyme phosphorylating glycogen from the enzymes converting Cori ester into glucose and Embden ester. By this elegant method they were able to show, in the clearest possible way, that the reaction $\text{glycogen} + \text{phosphate} \rightleftharpoons \text{Cori ester}$ is a true reversible equilibrium, about 82 % of the added Cori ester being converted into glycogen when equilibrium is attained.

This paper incidentally explains the great difficulty we experienced in preparing actively phosphorylating liver extracts; apparently, fasted rabbits, on which all our experiments were made, give much less active liver extracts than well-fed animals.

The results of Cori *et al.* differ from our own in one respect; they state, as the result of one experiment, that NaF has no effect on the formation of glucose or hexosemonophosphate from glycogen. We have performed a great number of experiments with NaF, and have never failed to observe a marked effect. This discrepancy may be due to the fact that the above authors used concentrations of NaF 40 times greater than those used in our experiments. The possibility also remains that the dephosphorylation of Cori ester in the intact liver, and in brei, may be brought about by a more complicated mechanism than the (apparently) simple phosphatase present in liver extracts.

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CCXXXI. DETERMINATION OF SMALL AMOUNTS OF SULPHUR AS SULPHATES IN BIOLOGICAL FLUIDS

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THREE main forms of sulphur are usually recognized in biological fluids: (a) inorganic sulphates, (b) ethereal sulphates and (c) neutral sulphur. The determination of these different forms of sulphur is usually made as sulphate. Several methods have been proposed but none of them is free from errors.

The earliest gravimetric methods depending on barium precipitation can only be used when large samples are available, as with urine. For blood sulphates barium precipitation has also been used, the precipitate being measured volumetrically [Hamburger, 1916; De Boer, 1917], or nephelometrically [Denis, 1921; Denis & Reed, 1926].

In other methods barium chromate is added and the liberated chromate estimated colorimetrically with diphenylcarbazide [Lang, 1929], or volumetrically [Klinke, 1924; Morgulis & Hemphill, 1932]. Øllgard [1934] estimated sulphates using an adsorption indicator (sodium rhodizonate).

An important advance was the use of benzidine proposed by Rosenheim & Drummond [1914] and applied by Raiziss & Dubin [1914] to the determination of sulphates in urine, and later to blood estimations by Fiske [1921]. Benzidine sulphate has been estimated by alkalimetric [Pohorecka-Lelesz, 1927; Cope, 1931; De Meio, 1933], iodimetric [Wakefield *et al.* 1931], manganimetric [Hoffman & Cardon, 1935] and colorimetric methods, the latter being based on conjugation reactions [Cuthbertson & Tompsett, 1931; Letonoff & Reinhold, 1936], or oxidation [Yoshimatsu, 1926; Hubbard, 1930; Wakefield, 1929].

All these methods are subject to error due to non-quantitative precipitation of benzidine sulphate and to the imperfection of the colour reactions employed. We therefore started the study of a new method, based on the use of benzidine as a precipitant but modifying the technique of precipitation so that complete removal of interfering substances was obtained. The benzidine sulphate is determined colorimetrically, using the weak reducing power of benzidine on phosphotungstomolybdic acid (Folin's phenol reagent). The Pulfrich photometer is employed for the final matching.

EXPERIMENTAL

Reagents.

Description of the method

Trichloroacetic acid: 10 % aqueous solution.

Ammonia solution: prepared by diluting 20 ml. of ammonia (sp. gr. 0.91) to 100 ml. and adding 5 ml. of 0.02 % methyl red.

Zirconium oxychloride or *aluminium chloride* ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$): 2.5 % aqueous solution. We have used both reagents but we prefer the aluminium salt because it is more easy to obtain pure. Commercial zirconium oxychloride contains much sulphate which must be eliminated before use. It can be purified using Archibald's [1932] method: the zirconium oxychloride is dissolved in conc. HCl,

precipitated by making weakly alkaline with ammonia and boiled for a while. The precipitate is washed with dilute (1%) HCl until free from sulphate. The washed precipitate is dissolved in hot conc. HCl and conveniently diluted.

The aluminium chloride used as a substitute for the zirconium salt can be obtained in a quite satisfactory degree of purity and used without previous purification.

Uranyl acetate: 0.4% aqueous solution.

Benzidine: 1.5% in anhydrous acetone. This solution is freshly prepared daily using pure benzidine and redistilled acetone.

Trichloroacetic acid: 5% in anhydrous acetone. This solution should be freshly prepared since it does not keep more than a few days; after this time a yellow tinge appears.

Hydrochloric acid: 0.2 N and N (both approximate).

Gum arabic: 1% solution. The gum arabic contains substances that reduce phosphotungstomolybdic acid, which can, however, be destroyed by oxidation with a few drops of bromine; before use, an adequate amount of the solution is boiled until completely free from bromine.

Phosphotungstomolybdic acid reagent: prepared according to Folin & Ciocalteu [1927].

Sodium carbonate: 16% solution, prepared from the anhydrous salt of good quality.

Sodium sulphite: 2% solution, must be prepared every day.

Benzidine hydrochloride: weigh 4.0068 g. of pure and dry benzidine hydrochloride, transfer to a 1000 ml. flask and dissolve in 0.2 N HCl to volume. Each ml. of this solution is equivalent to 0.5 mg. of sulphur. This solution keeps indefinitely.

As standard, a dilute solution is prepared by diluting 1 ml. of the concentrated solution to 50 ml. with 0.2 N HCl. Each ml. of this diluted standard corresponds to 0.010 mg. (10 μ g.) of sulphur. The diluted solution does not keep more than 2-3 days.

Glassware must be scrupulously clean and free from sulphates. As cleaning solution we use a mixture of 300 ml. of conc. HNO_3 with 1000 ml. of 10% $\text{K}_2\text{Cr}_2\text{O}_7$.

Collection of blood and urine. The determination of sulphur may be carried out in whole blood, blood plasma or serum. For the determination in whole blood or blood plasma an anticoagulant must be used. As shown by Pohorecka-Lelesz [1927], potassium oxalate or sodium fluoride cannot be used since they precipitate with benzidine. Sodium citrate can only be used when the separation of the interfering substances with aluminium chloride or zirconium oxychloride is omitted. The best anticoagulant which can be used is heparin (see p. 1882, however). No special precautions are necessary for collecting the urine.

The sulphur of the blood

Inorganic sulphates. To 1 vol. of whole blood, serum or plasma add 2 vol. of 10% trichloroacetic acid. Filter after 10-20 min. In a 15 ml. conical centrifuge tube measure 6 ml. of filtrate and add 0.5 ml. of 2.5% aluminium chloride (or zirconium oxychloride) solution, thoroughly mix with a very thin glass rod and add 0.5 ml. of 20% ammonia with methyl red. The solution must be distinctly alkaline. Let stand for a while, until aluminium hydroxide is flocculated. Centrifuge 5 min. at 2000 r.p.m. Any considerable excess of ammonia must be avoided, because some of the aluminium hydroxide may redissolve and be reprecipitated when the solution is acidified.

In another 15 ml. conical centrifuge tube, with a fine (2–3 mm.) end, put 3.5 ml. of the clear centrifuged solution and neutralize by adding 5% trichloroacetic acid in acetone; about 1 ml. is necessary. Add 2 ml. more of the trichloroacetic acid in order to make the liquid distinctly acid and finally 6 ml. of 1.5% benzidine solution in acetone.

Mix with the aid of a thin glass rod and keep in the ice box at 0° for 2 hr. or more. Centrifuge at about 3000 r.p.m. for 15 min.

The supernatant fluid is decanted by inverting the tubes and allowing them to drain for 5 min. on a filter paper moistened with acetone.

After this time, the precipitate is washed at the centrifuge with 10 ml. of acetone containing 5% trichloroacetic acid, and the supernatant fluid is decanted and drained as before. The washed precipitate is dissolved in 2 ml. of 0.2 N HCl by heating in a water bath for a few minutes.

The solution is transferred quantitatively to a 25 ml. graduated tube with the aid of 3 or 4 washings with small quantities of water (1 or 2 ml.). The final volume must be about 6–8 ml.

The colour reaction is developed by adding the following reagents in the order specified and thoroughly shaking after each addition: add 0.5 ml. of gum arabic solution and 1 ml. of phosphotungstomolybdic acid reagent; let stand 3 min. or more, add 2.5 ml. of 16% Na_2CO_3 and let stand at least 10 min.; finally add 2 ml. of recently prepared 2% sodium sulphite; after a few minutes add water to make 20 ml.

The blue-grey colour produced is compared with a standard prepared simultaneously as follows. In two similar graduated test tubes put 1 ml. and 2 ml. of the standard benzidine solution equivalent to 10 and 20 μg . of sulphur, add water to make 6 or 8 ml., followed by the reagents in the above-mentioned order.

The determination may be made with greater accuracy in a Pulfrich photometer using filter S. 72 (see p. 1888).

Total sulphates. In a small beaker put 6 ml. of blood filtrate and 0.5 ml. of N HCl; heat the beaker in a water bath until dry. Let cool and dissolve the residue in 6 ml. of distilled water, add 0.5 ml. of 2.5% aluminium chloride and 0.5 ml. of 20% ammonia. Centrifuge, take 3.5 ml. of the clear supernatant fluid, equivalent to 1 ml. of blood, and carry out the determination as indicated for inorganic sulphates.

The sulphur corresponding to the ethereal sulphates is calculated from the difference between that of the total and inorganic sulphates.

Total sulphur. In a 100 ml. Kjeldahl flask put 3 ml. of blood filtrate and add 3 ml. of pure conc. HNO_3 . Heat over gauze, with a Folin's fume absorber fitted to the flask, until the liquid turns brown and add a few drops of perhydrol. Continue the heating until a white ash is obtained. Let cool and add 6 ml. of distilled water, 2.5 ml. of aluminium chloride solution and 0.5 ml. of ammonia; centrifuge and complete the determination as for inorganic sulphates upon 3.5 ml. of the clear supernatant fluid, equivalent to 0.5 ml. of blood.

The neutral sulphur is calculated from the difference between the value obtained and that of the total sulphates-sulphur.

The sulphur of the urine

Preparation of the urine. The urine must be conveniently diluted and the interfering substances removed. Phosphates are the most important interfering substances in urine and can be removed according to Letonoff & Reinhold [1936] with uranyl acetate.

The greater the volume of urine excreted, the greater must be the quantity taken for the analysis. For urine of normal concentration, 1 ml. is transferred to a 50 ml. volumetric flask and 4 ml. of 0.4 % uranyl acetate are added. The flask is thoroughly shaken and kept for 5 min. or more. At the end of this time the mixture is diluted to volume and filtered. This filtrate is used in all the determinations.

Inorganic sulphate. In a 15 ml. conical centrifuge tube put 1 ml. of filtrate equivalent to 0.02 ml. of urine and add 2 ml. of 10 % trichloroacetic acid. The rest of the procedure is as indicated for blood.

Total sulphate. The determination is carried out on 0.5 ml. of filtrate equivalent to 0.01 ml. of urine; this is treated with 2-3 ml. of water and 0.5 ml. of *N* HCl and further treated as described for blood.

Total sulphur. 1 ml. of filtrate is incinerated with HNO_3 and perhydrol as described above; the ash is dissolved in 6 ml. of 10 % trichloroacetic acid and 3 ml., equivalent to 0.01 ml. of urine, are taken for the analysis.

NOTES ON THE METHOD

Action of anticoagulants

The determination of sulphur in whole blood or blood plasma needs the use of an anticoagulant to prevent blood clotting. As pointed out above oxalates cannot be used and citrates can only be employed when the removal of interfering substances by aluminium chloride or zirconium oxychloride is omitted. Heparin is a good anticoagulant for inorganic sulphate determination, but is useless for ethereal and total sulphur determinations, because it contains sulphate which is set free during the hydrolysis or the destruction of the organic matter. Defibrination is quite satisfactory.

Deproteinization of the blood

Of the several deproteinizing reagents which have been proposed for sulphur determinations we have found trichloroacetic acid to be the most satisfactory when used in the manner specified.

When interfering substances are not removed, the quantity of trichloroacetic acid employed provides a distinctly acid filtrate (*pH* 2.8-3) of the most convenient acidity for the precipitation of sulphates with benzidine without simultaneous precipitation of benzidine phosphate.

When aluminium is used for removing interfering substances the filtrate is made alkaline and after separation of the aluminium hydroxide the liquid must therefore be reacidified with trichloroacetic acid-acetone before precipitating with the benzidine reagent.

Table I. *Comparison of the Letonoff-Reinhold and trichloroacetic acid techniques for deproteinization*

	Inorganic sulphates in mg. per 100 ml.	
	Trichloroacetic acid	Uranyl acetate
Human serum	1.45	1.54
	1.40	1.38
	1.60	1.56
Dog serum	4.97	4.91
	5.06	5.00

The deproteinizing technique with uranyl acetate, proposed by Letonoff & Reinhold [1936], offers no advantages over that used by us. Comparative results of both deproteinizing techniques upon the same blood sample are given in Table I. The results show little difference between the two methods. We have been unable to confirm the statement of Letonoff & Reinhold, that in trichloroacetic acid filtrates the inorganic sulphates increase on standing. We have analysed the same acid blood filtrate several days in succession without observing any alteration in the inorganic sulphate content.

Time of precipitation

The best conditions for complete precipitation of benzidine sulphate are a temperature of about 0° and a minimum time of about 1 hr.; longer times up to 24 hr. do not affect the results.

Washing of the precipitate

In contradiction to Hubbard's [1930] statement, we have found that neutral acetone dissolves an appreciable quantity of benzidine sulphate. Letonoff & Reinhold [1936], on the other hand, have found that the addition of 10% of water to the acetone used in the washing produces a loss of sulphate-sulphur varying between 20 and 50%.

We have found that acidifying the acetone used for washing with 5% trichloroacetic acid gives satisfactory results, as can be seen from the comparative figures shown in Table II.

Table II. *Effect of washing the precipitate of benzidine sulphate*

Washing liquid	S as H ₂ SO ₄ μg.	S found μg.	Error %
Neutral acetone	10	9.30	- 7.0
	10	8.69	- 13.1
	10	7.54	- 24.6
	10	9.95	- 0.5
Acetone plus 5% trichloroacetic acid	10	9.75	- 2.5
	10	10.00	0.0
	10	10.00	0.0
	10	10.00	0.0

Removal of interfering substances

Several workers have recognized that the principal cause of error in sulphate determinations with benzidine is the presence of phosphates and chlorides.

The phosphates precipitate with benzidine as benzidine phosphate, particularly when the reaction of the solution is neutral or weakly acid. Fiske [1921] tried for the first time the separation of phosphates from sulphates with little success. When the quantity of phosphates is small their precipitation can be avoided by acidifying the solution to pH 2.5 or 3. In this way, sulphates of serum or plasma can be determined with a good degree of accuracy. Whole blood sulphates cannot be determined in the same way, because of the greater concentration of phosphates and other interfering substances.

For the removal of phosphates we first used the technique of Curtman *et al.* [1924] with zirconium oxychloride and later replaced it by the aluminium chloride method with similar results. The influence of phosphates upon sulphur determinations in pure mixtures of sulphates and phosphates with the benzidine reagent has been studied by us, with the results shown in Table III, where the effectiveness of the phosphate removal by zirconium oxychloride can be seen.

Table III. *Interference of phosphates in sulphate determination*

Mixture used		With phosphate removal by zirconium oxychloride		Without phosphate removal	
S as H_2SO_4 $\mu\text{g.}$	P as H_2PO_4 $\mu\text{g.}$	S found $\mu\text{g.}$	Error %	S found $\mu\text{g.}$	Error %
10	50	9.9	-1.0	12.90	+ 29.0
10	50	10.0	0.0	24.10	+ 141.0
10	100	9.9	-1.0	15.38	+ 54.0
10	100	10.0	0	12.50	+ 25.0

The results show that phosphates interfere with sulphur determination when their concentration is 5 times greater, or more, than that of sulphate. For smaller quantities of phosphates the interference is not appreciable because the trichloroacetic acid-acetone used in the washing removes practically all the benzidine phosphate.

The effectiveness of zirconium oxychloride and aluminium chloride in the removal of interfering substances is demonstrated in the experiments of Table IV

Table IV. *Recovery of sulphur added to dog's blood treated with zirconium oxychloride or aluminium chloride*

Precipitating agent	S in the blood sample $\mu\text{g.}$	S added $\mu\text{g.}$	S calculated $\mu\text{g.}$	S found $\mu\text{g.}$	Error %
Zirconium oxychloride	20.50	—	20.50	20.50	—
	20.50	10	30.50	31.44	+ 3.0
	20.50	20	40.50	39.80	- 2.2
Aluminium chloride	17.34	—	17.34	17.34	—
	17.34	10	27.34	27.05	- 1.1
	17.34	20	37.34	36.54	- 2.2

where known quantities of sulphur as sulphate were added to blood. Recovery of sulphates is quantitative with an error not greater than 3%. The equal efficiencies of zirconium oxychloride and aluminium chloride in removing the interfering substances were proved by treating two samples of the same blood filtrate with each of them and determining the sulphur (Table V).

Table V. *Efficiencies of zirconium and aluminium in removing interfering substances from blood plasma*

Mg. of inorganic sulphur per 100 ml.	
Zr treatment	Al treatment
3.43	3.41
3.43	3.37

Experiments carried out with blood without removing interfering substances give very high values for inorganic sulphate as shown in Table VI.

Table VI. *Determination of inorganic sulphur in blood, with and without the removal of interfering substances*

Dog's blood sample	Mg. of inorganic sulphur per 100 ml.		Increase produced by interfering substances %
	Without removing interfering substances	Removing interfering substances	
1	8.32	3.93	111
2	9.45	3.77	150
3	5.60	2.85	96

The acid-acetone used for washing removes practically all the benzidine phosphate of the precipitate so that some other unknown substances not removed by acid-acetone must be precipitated with the benzidine sulphate in the direct determination. This hypothesis is confirmed by the following facts: the results presented in Table III show that 100 μ g. of phosphorus as phosphate produce an error from +25 to +50 % in the sulphur determination, whilst in blood filtrates, with much less phosphate, the error is from +100 to +150 %.

In order to prove the existence of interfering substances other than phosphates, the following experiments were made.

Exp. 1. In a sample of blood, sulphates were precipitated without removing the interfering substances by the zirconium or aluminium treatment. The precipitate, washed once with neutral acetone, was dissolved by adding ammonia and heating. After cooling the benzidine was extracted with ether. The liquid, freed from ether by heating, was divided into two portions. One was analysed for phosphate by Fiske & Subbarow's method and with the strychnomolybdic reagent. No inorganic phosphorus could be detected. The other portion was ashed and the analysis for phosphorus gave positive results. This means that some organic phosphorus compound was precipitated along with the sulphates by the benzidine reagent, and retained by the precipitate without being removed by the neutral acetone used as washing fluid.

This experiment proves that the principal interfering substance or substances are organic compounds and that they contain phosphorus in combined form.

Exp. 2. A sample of blood was deproteinized with trichloroacetic acid and divided into three portions:

(a) One was precipitated directly and inorganic sulphates plus interfering substances determined. It gave 13.33 mg. S per 100 ml.

(b) The second fraction was precipitated with benzidine in the same manner. The precipitate was ashed and the residue dissolved and reprecipitated with benzidine. It gave 4.65 mg. S per 100 ml.

(c) The third fraction was treated with aluminium chloride to remove the interfering substances and sulphates precipitated with benzidine. It gave 4.56 mg. S per 100 ml. These results clearly demonstrate that the benzidine precipitate, ashed and reprecipitated, gave the same value as the direct determination after aluminium treatment.

The interfering substances are therefore destroyed during the ashing process and must be of organic nature.

These results speak for the great specificity of the benzidine as a sulphate precipitant when interfering substances had been previously removed.

The colour reaction

Some investigators who have used colorimetric determination of benzidine have employed oxidation reactions which are useless because of their uncontrollability. Others have employed azo derivatives of benzidine which are also useless because of their sparing solubility and lack of strict proportionality between colour and concentration of benzidine. Nobody, as far as we are aware, has hitherto employed reactions based on the weak reducing power of benzidine.

The colour produced between Folin's phenol reagent and benzidine is green at first and turns blue-green on standing for a long time. The reaction does not fade, it is proportional and suited for quantitative colorimetric analysis. As the reaction is carried out in alkaline solution a fraction of the phosphotungstomolybdic acid reagent is reduced by the benzidine sulphate, and the excess of reagent is completely destroyed by the alkali. This fact has permitted us to

bring the indefinite green colour of the reaction to a blue-grey stable colour by adding sodium sulphite after destruction of the excess of phenol reagent by alkali. This is the reason why 10 min. should elapse before adding the sodium sulphite which brings the colour to a definite maximum.

The colour obtained is blue-grey and not a pure blue, as in the direct reduction of phosphotungstomolybdic acid. The difference is due to the fact that oxidized benzidine forms a brown compound (probably a meriquinone), which remains in solution for some time, altering the blue colour of the reduced phenol reagent. As this compound precipitates on standing, we have been obliged to use as a protective colloid the gum arabic solution. Several previous trials demonstrated that 0.5 ml. of gum arabic solution, previously oxidized with bromine, is sufficient to prevent the precipitation of a quantity of benzidine 2 or 3 times larger than that present in the standard solution, and with the production of a blank reaction practically negligible for colorimetric determination.

When the photometric method is used, the quantity of gum arabic added may be greater if the blank used as compensating fluid is made with the same quantity of gum solution.

The action of sodium carbonate

The quantity of sodium carbonate to be added must be properly adjusted by previous titration of the phenol reagent. A deficit of sodium carbonate leaves excess of phenol reagent and the addition of sodium sulphite will reduce this excess to give more colour. An excess of sodium carbonate destroys the benzidine colour as may be seen in Table VII.

Table VII. *Influence of sodium carbonate*

Standard benzidine solution equivalent to S μg.	Sodium carbonate 16% ml.	Colorimetric reading mm.
10	2.0	13.2
10	2.2	17.2
10	2.3	19.9
10	2.4	20.1
10	2.5	20.0
10	2.6	20.0
10	2.7	20.1
10	2.8	20.0
10	3.0	21.1
10	3.3	22.0

For the titration of the phenol reagent the following procedure is used.

Quantities of standard benzidine solution equivalent to 10 μg. of sulphur are put in a series of graduated tubes; all the reagents are added as usual, except the sodium carbonate, which is added in increasing amounts from 2 to 3 ml. as is shown in Table VII. As can be seen from this table there is a zone of sodium carbonate concentration where the colorimetric reading is constant (in this case from 2.3 to 2.8 ml.); the mean value is taken as the optimal concentration of sodium carbonate to be added for 1 ml. of phenol reagent. 1 ml. of phenol reagent is usually enough and there is no advantage in using more. It suffices for more than 20 times the quantity of benzidine used as standard.

Influence of sodium sulphite

This reagent is employed to complete the reduction of that portion of the phenol reagent which is partly reduced by the benzidine. If the excess of phenol reagent has been destroyed with the proper quantity of sodium carbonate, excess

of sodium sulphite is not harmful. The usual quantity of sodium sulphite added, 2 ml. of a 2 % solution, is more than enough for complete reduction of the reagent and the excess stabilizes the colour for long periods of time.

Proportionality of the colour reaction

The proportionality between the coloured reaction and sulphur concentration has been determined colorimetrically with the results expressed in Table VIII.

Table VIII. *Proportionality of the colour reaction*

Standard benzidine solution equivalent to S μg.	Theoretical reading mm.	Actual reading mm.		
		I	II	III
5	40.0	39.8	39.6	40.0
10 (test)	20.0	20.0	20.0	20.0
20	10.0	10.1	10.0	10.1
10	40.0	39.8	40.0	40.3
20 (test)	20.0	20.0	20.0	20.0
40	10.0	10.0	10.0	9.9

If the unknown is too dark to be compared with the standard, it can be diluted with water without change in the proportionality. Strict proportionality of the reaction has also been found in the photometric determination of the colour with the Pulfrich photometer (see p. 1888).

Accuracy of the method

The method was first checked by determination of a pure sulphuric acid solution, previously analysed by the barium sulphate method, and conveniently diluted for the assays. The results obtained are given in Table IX. Tables X and XI show the accuracy with which added sulphate may be recovered from blood and urine respectively.

Table IX. *Analysis of pure sulphuric acid solution*

S as H ₂ SO ₄ μg.	S found μg.	Error %	S as H ₂ SO ₄ μg.	S found μg.	Error %
5	5.00	0.0	10	10.05	0.5
5	4.95	-1.0	10	9.95	-0.5
5	5.00	0.0	15	14.70	-2.0
5	5.00	0.0	15	14.80	-1.3
8	7.87	-1.6	16	16.00	0.0
10	9.90	-1.0	20	19.60	-2.0
10	9.85	-1.5	20	20.00	0.0
10	10.00	0.0	20	19.80	-1.0

Table X. *Recovery of inorganic sulphates from dog's blood*

S in 1 ml. of serum μg.	S added μg.	S calculated μg.	S found μg.	Error %
14.38	5.0	19.38	19.23	-0.7
14.18	10.0	24.18	24.08	-0.4
17.39	5.0	22.39	21.96	-1.9
16.53	7.5	24.03	23.82	-0.9
16.53	22.5	39.03	37.50	-3.9
12.96	15.0	27.96	27.42	-1.9

Table XI. *Recovery of sulphates added to urine*

S in 1 ml. of urine μg.	S added μg.	S calculated μg.	S found μg.	Error %
555.5	250	805.5	770.0	-4.4
555.5	500	1055.5	1041.5	-1.3
570.5	250	824.5	819.5	-0.6

Photometric determination

The coloured solution does not possess a typical maximum absorption, but extinction increases steadily from the violet to the red portion of the spectrum; the most suitable filter is S. 72. As compensating liquid a blank is used with all the reagents in the same proportions as in the assay. The thickness of the layer used under ordinary conditions was 10 mm. but for concentrated solutions a smaller vessel can be used.

With the aid of the standard benzidine solution we have determined the calibration curve shown in Table XII.

Table XII. *Photometric calibration curve of S as benzidine equivalent*

Standard benzidine solution as S μg.	Extinction for 1 cm. layer	Specific extinction coefficient for 1 mg.
5	0.201	40.2
10	0.391	39.1
15	0.588	39.1
20	0.775	38.7
30	1.164	38.8
40	1.586	39.6
	Mean value	39.25

The photometric determination of sulphates presents advantages over the colorimetric method, not only because it is more accurate and requires no standard, but also because a larger amount of gum solution can be added to prevent the formation of the precipitate when high concentrations of sulphur are present.

The specific extinction coefficient for 1 mg. of sulphur was calculated from the analytical data obtained (Table XII) whose value is $E_{1\text{ cm.}} = 39.25$. Its constancy for different concentrations of benzidine equivalent to sulphur speaks for the strict proportionality of the reaction.

SUMMARY

A new micro-method for the estimation of the several known forms of sulphur in blood and urine is described. Quantities of sulphur of 5 μg. can be estimated with an error of 3%.

Interfering substances are precipitated with aluminium, the sulphates with benzidine and the latter is estimated colorimetrically or photometrically by the reduction of phosphotungstomolybdic acid.

It is suggested that substances other than phosphates interfere in the usual methods of sulphur determination with benzidine.

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CCXXXII. MAGNESIUM AND MUSCLE RESPIRATION

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SZENT-GYÖRGYI and his school first emphasized the importance of the dicarboxylic acids, succinic, fumaric, malic and oxaloacetic, in the respiration of minced pigeon breast muscle. They indicated that these compounds acted as H carriers between the oxidizable substrates present in their preparations and the cytochrome system. This conception was placed on a firm basis by the work of Stare & Baumann [1936] who showed that these dicarboxylic acids acted in a truly catalytic manner.

In the past two years this theory has been extended by Krebs and his co-workers [Krebs & Johnson, 1937; Krebs & Eggleston, 1938]. They showed that citric acid and its decomposition products, *cis*aconitic acid, *isocitric* acid and α -ketoglutaric acid, exerted a similar effect to the dicarboxylic acids mentioned above; further, they have shown that citric acid is synthesized from oxaloacetic acid and an unknown precursor referred to as triose, though Breusch [1937] has contested this latter claim. On the basis of this work Krebs has postulated the existence, in minced muscle preparations, of a citric acid cycle in which citric acid is first synthesized from oxaloacetic acid and the carbohydrate breakdown product triose, the citric acid so formed is then oxidized to CO_2 and oxaloacetic acid, the net effect being the oxidation of triose. This attractive theory of the oxidation of carbohydrate by muscle is supported by the fact that insulin stimulates the utilization of O_2 by these preparations in the presence of citric acid or its breakdown products [Krebs & Eggleston, 1938] and it also provides an explanation of the CO_2 production.

That boiled muscle or yeast extracts exert a stimulating action on the respiration of muscle has been known for some time, the action being ascribed to the presence of coenzymes and substrates. Greville [1937] showed that fumaric acid, or Mg or coenzyme I, would increase the O_2 consumption of a dispersion of pigeon breast muscle in phosphate buffer of pH 7.3; he also demonstrated that boiled yeast extract exerted a stimulating action on his preparation. The present paper is an attempt to determine to what extent the activity of boiled muscle extract is due to the Mg or dicarboxylic acids it contains.

Methods

Material. Pigeon breast muscle was used throughout. The pigeons were killed by decapitation and the pectoral muscles dissected out and cooled in ice. The muscles were then minced in a Latapie mincer which had been cooled in the ice chest. The mince was suspended in $M/10$ phosphate buffer, pH 7.4, and 2 ml. were used in each experiment. The boiled muscle extract was made from sheep's heart by the method of Krebs & Eggleston [1938] and the reaction was adjusted to pH 7.4 before use.

O_2 consumption was measured by means of the Warburg manometric apparatus. CO_2 was absorbed by 0.3 ml. of 10% NaOH placed in the centre tube

which was fitted with a folded filter paper. All additions were made from the side bulb which, in all cases, contained 1 ml. of fluid, making the total volume in which the tissue was suspended 3 ml. The experiments were carried out in an atmosphere of O_2 .

For the estimation of succinic and fumaric acids the ethereal extraction described by Elsdon [1938] was used; the succinic acid was estimated by the manometric method of the same author. The estimation of fumaric acid was achieved by a microhydrogenation technique using colloidal Pd as a catalyst, the volume of H_2 absorbed being measured manometrically. The details of this method were worked out by Mr Kenneth Harrison of the Biochemical Laboratory, Cambridge.

Mg was estimated by the oxyquinolate method of Greenberg *et al.* [1935]. Mg was added in the form of the chloride and the strength of the solution was checked by estimation before use. Succinic and fumaric acids were added as the Na salts.

EXPERIMENTAL

The effect of added Mg. Addition of Mg alone to minced muscle caused a slight increase in the amount of O_2 used. The addition of succinate or fumarate alone, as was known from earlier work, produced an increase in O_2 uptake, but in the present experiments the effect was not always catalytic. This discrepancy can be explained in terms of H ion concentration, for Krebs & Eggleston found that the catalytic effect of these substances is more apparent at pH 6.8 than at 7.4, the reaction at which these experiments were carried out. The addition of both a dicarboxylic acid and Mg brought about a large increase in the total O_2 consumed. The effect was not simply additive, but was in all cases greater than the sum of the effects of either substance acting alone. The results using fumaric and succinic acids are summarized in Tables I and II respectively. It will be

Table I. O_2 uptake in presence of fumarate and Mg

0.37 mg. fumaric acid was added as Na salt. Figures in parentheses represent the wt. of Mg added in mg.

	Dilution of muscle	Time min.	O_2 uptake (μ l.)				
			Blank	\pm Fum.	+ Mg	Fum. + Mg	
1	1:10	90	631	990	786	1222	(0.31)
2	1:10	90	571	977	686	1172	(0.31)
3	1:10	90	613	840	691	1171	(0.31)
4	1:10	90	839	1011	850	1532	(0.62)
5	1:10	120	851	1241	1047	1653	(0.31)

Table II. O_2 uptake in presence of succinate and Mg

0.38 mg. succinic acid added as Na salt, and 0.31 mg. Mg added as chloride.

Dilution of muscle	Time (min.)	O_2 uptake (μ l.)			
		Blank	\pm Succinate	+ Mg	Succinate + Mg
1:10	120	1117	1337	1151	1651
1:10	150	724	970	883	1495
1:20	150	357	440	426	732

seen that where 0.62 mg. Mg was used the effect was certainly not additive; this is also demonstrated in Fig. 1. Table III shows the effect of varying the amount of Mg added, and it will be seen that the magnitude of the effect increases with

increasing amounts of Mg up to 0.62 mg., but that a higher concentration—1.55 mg.—while producing an increase in O_2 uptake over and above the control, is less effective than 0.62 mg. This inhibitory effect was first observed by Greville [1937].

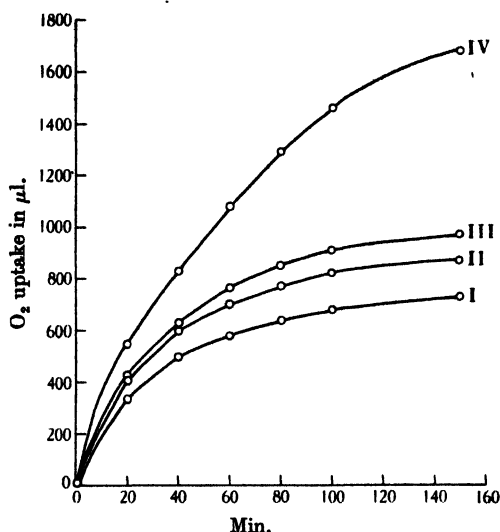


Fig. 1.

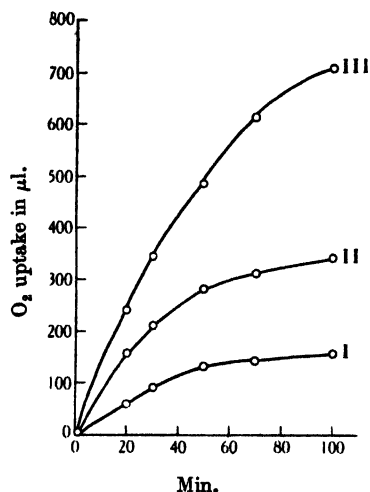


Fig. 2.

Fig. 1. Effects of succinic acid and Mg on the respiration of minced pigeon breast muscle diluted 1:10 with phosphate buffer pH 7.4. I, control; II, +0.39 mg. succinic acid; III, +0.62 mg. Mg; IV, 0.39 mg. succinic acid +0.62 mg. Mg.

Fig. 2. Effect of boiled muscle extract compared with equivalent amounts of Mg and succinic acid on the respiration of minced pigeon breast muscle diluted 1:20 with phosphate buffer pH 7.4. I, control; II, +0.1 mg. Mg +0.22 mg. succinic acid; III, +1 ml. of boiled muscle extract.

Table III. O_2 uptake with varying concentrations of Mg

0.37 mg. of fumaric acid added as Na salt present in all cases.

Dilution of muscle	Time min.	O_2 uptake (μ l.)				
		Control	0.002 mg. Mg	0.31 mg. Mg	0.62 mg. Mg	1.55 mg. Mg
1:5	85	918	—	1598	2381	2028
1:5	90	824	973	1602	2163	1924
1:10	120	1024	—	1963	1971	1916
1:10	120	1232	—	1443	1816	1635
1:10	90	973	—	1457	1543	1230

The effect of boiled muscle extract. Before use the extract was assayed for Mg, fumaric acid and succinic acid, and for comparison a solution of succinic acid was used equivalent to the total dibasic acids present in 1 ml. of the extract; Mg was added to this solution in an amount corresponding to that in 1 ml. of extract. The results obtained are shown in Fig. 2. It will be seen that the activity of boiled muscle extract exceeds the activity of an equivalent amount of succinic acid and Mg. Boiled muscle extract produced a 350% increase in the O_2 used whereas the succinic acid-Mg mixture caused an increase of 120%. In the experiment quoted 0.1 mg. Mg was used whereas the extract contained only 0.087 mg., a circumstance operating in favour of the mixture.

The effect of iodoacetic acid. In view of the known function of Mg as a co-phosphorylase in glycolysing systems the possibility existed that it was playing the same role in the respiration of the muscle preparations described above and, therefore, that part at least of the glycolytic system was functioning here. In order to determine the extent to which this occurred under the aerobic conditions

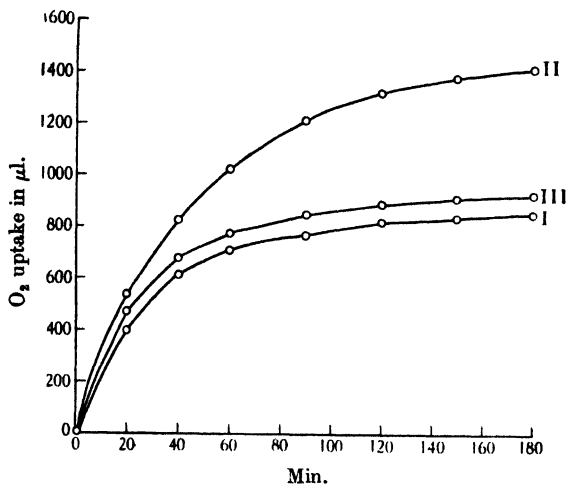


Fig. 3. Effect of iodoacetic acid on the respiration of minced pigeon breast muscle diluted 1:10 with phosphate buffer pH 7.4. I, control; II, +0.39 mg. succinic acid +0.62 mg. Mg; III, same as II but with $M/1000$ iodoacetic acid.

of the experiment, iodoacetic acid, in a final concentration of $M/1000$ was added. The results are expressed in Fig. 3. It will be observed that iodoacetic acid gives almost complete inhibition of the succinic acid-Mg effect after a latent period of about 30 min.

DISCUSSION

It is clear from these experiments that the stimulating action of boiled muscle extract on muscle respiration cannot be explained on the basis of its Mg and dicarboxylic acid contents alone, though it is certain that these are essential components of the system. From the work of Greville [1937] it is certain that coenzyme I plays a part, but as no method for the estimation of this substance was available, it was impossible to obtain a quantitative idea of its importance. It is also conceivable that other coenzymes are involved.

The action of the succinic and fumaric acids can be explained on the Szent-Györgyi hypothesis or the citric acid cycle, but the action of Mg is not so clear. Lohmann [1931] showed that Mg formed an essential component of the glycolysing system found in muscle extracts where it acted as a co-phosphorylase. Recently, Adler *et al.* [1939] have studied the effect of Mg on the isocitric dehydrogenase present in heart muscle and an essential component of the citric acid cycle, and have shown that it is an activator of this enzyme.

On this evidence there are three interpretations of the Mg effect: (a) That it is acting solely as a co-phosphorylase and therefore its presence stimulates the production of triose, the substance oxidized. (b) That it is acting in the capacity of activator to the isocitric dehydrogenase. (c) That Mg acts in both these ways. In the absence of further evidence this last possibility seems to be the most reasonable.

Krebs [1931] showed that $M/1000$ iodoacetic acid completely inhibited the O_2 uptake in the presence of glucose but not of lactic acid, by slices of rat sarcoma, rat brain cortex and rat testis. Since then iodoacetic acid has been shown to inhibit two mammalian dehydrogenases: the triosephosphate dehydrogenase [Green *et al.* 1937] and the isocitric dehydrogenase [Adler *et al.* 1939]. The exact point at which iodoacetic acid acts in the muscle system is not yet clear. It may be inhibiting the triosephosphate dehydrogenase or the isocitric dehydrogenase or both. Further work is in progress to attempt to elucidate this point.

SUMMARY

1. Mg, especially in the presence of succinic or fumaric acids, stimulates the respiration of minced pigeon breast muscle.
2. The stimulating action of boiled muscle extract on the respiration of minced pigeon breast muscle cannot be completely accounted for by its Mg and dicarboxylic acid contents.
3. $M/1000$ iodoacetic acid completely inhibits the succinic acid-Mg effect after a latent period of 30 min.

The author wishes to express his thanks to Mr Kenneth Harrison of the Biochemical Laboratory, Cambridge, for the details of the method for estimating fumaric acid, and to the Moray Fund of Edinburgh University which defrayed the expenses of this research.

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CCXXXIII. METABOLISM OF α -KETOGLUTARIC ACID IN ANIMAL TISSUES

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DEWAN [1938] and Adler *et al.* [1939] reported that certain enzyme preparations obtained from heart muscle bring about a reductive amination of α -ketoglutaric acid. The hydrogen required in this reaction may be provided from other dehydrogenase systems via coenzyme I or II, especially from the β -hydroxybutyric and isocitric dehydrogenases, according to the schemes:

(1) α -ketoglutaric acid + NH_3 + isocitric acid \rightarrow glutamic acid + oxalosuccinic acid, or

(2) α -ketoglutaric acid + NH_3 + β -hydroxybutyric acid \rightarrow glutamic acid + acetoacetic acid.

We have studied the occurrence of these reactions (so far observed only in artificially combined enzyme systems) in surviving tissues. We find that a reductive amination of α -ketoglutaric acid does in fact take place in kidney and heart muscle, but this reduction proves independent of the presence of isocitric or β -hydroxybutyric acids. On the other hand it is found to be accompanied by a formation of succinic acid and CO_2 , and the quantities of these substances formed show that the following reaction takes place:

(3) 2α -ketoglutaric acid + NH_3 = glutamic acid + succinic acid + CO_2 .

EXPERIMENTAL

Pigeon breast muscle and heart muscle were minced in the Latapie mill and suspended in 5 to 10 parts of 0.1 *M* phosphate buffer of pH 7.1 or 7.4. Other tissues were sliced and suspended in the balanced salt solution of Krebs & Henseleit [1932]. The experiments were carried out in conical manometer flasks provided with sidearms and centre cups. The latter contained yellow P in all anaerobic experiments. The temperature of the bath was 40°.

Glutamic and succinic acids were determined manometrically as previously described [Cohen, 1939]. The CO_2 production was also measured manometrically. The figures given in the tables for the CO_2 formation represent the total CO_2 , i.e. CO_2 formed from α -ketoglutaric acid and liberated from the NaHCO_3 of the medium by acids.

α -Ketoglutaric acid and ammonia in kidney cortex

In Table I two experiments are recorded in which kidney cortex was incubated anaerobically with α -ketoglutarate and NH_4Cl . It will be seen that small quantities of glutamic acid are formed when α -ketoglutarate alone is added. Addition of NH_4Cl increases the glutamic acid formation and at the same time approximately equivalent quantities of succinic acid and CO_2

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Table I. α -Ketoglutaric acid and NH_3 in kidney cortex

Species	Substrate added (final conc.)	Mg. tissue	Period of in- cubation min.	$\mu\text{l.}$ CO_2 evolved	$\mu\text{l.}$ succinic acid formed	$\mu\text{l.}$ glutamic acid formed
Guinea-pig	0.02 M α -ketoglutarate	28.8	100	—	—	122
	0.02 M α -ketoglutarate, 0.02 M NH_4Cl	39.7	100	—	—	518
	—	—	—	—	—	—
Rat	—	13.5	140	74	0	0
	0.02 M α -ketoglutarate	17.1	140	136	80	81
	0.02 M α -ketoglutarate, 0.02 M NH_4Cl	17.9	140	327	322	234

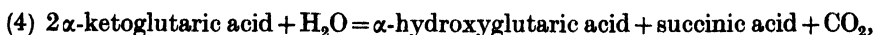
appear. In the experiment on rat kidney the ratio of glutamic acid : succinic acid : CO_2 is 234 : 222 : 229, or 1 : 0.95 : 0.98. These data are in agreement with equation (3).

Table II. Anaerobic disappearance of NH_3 in guinea-pig kidney cortex in the presence of α -ketoglutarate

(Total volume of medium per flask 4.6–4.9 ml.; incubation 195 min.; initial concentration of α -ketoglutarate 0.026 M)

$\mu\text{l. NH}_3$ added (as NH_4Cl)	mg. tissue	$\mu\text{l. NH}_3$ found after incubation	$\mu\text{l. NH}_3$ used per mg. tissue	$\mu\text{l. CO}_2$ formed per mg. tissue	"Extra CO_2 " (due to addition of NH_3)	"Extra CO_2 " formed — NH_3 used
—	78.5	—	—	5.68	—	—
1320	70.2	382	13.38	16.62	10.94	0.82
660	130.0	120	4.16	8.65	2.97	0.71
330	104.2	86.5	2.34	7.89	2.21	0.95

Table II shows a disappearance of NH_3 of the expected magnitude. At a low NH_3 concentration the "extra CO_2 " (i.e. CO_2 formed on addition of NH_3) is very nearly equivalent to the quantity of NH_3 absorbed. With rising concentrations of NH_3 the calculated "extra CO_2 " becomes smaller than the NH_3 absorbed. This may be explained on the assumption that a "simple" dismutation of α -ketoglutaric acid (reaction (4))



which accounts for most of the CO_2 formation from α -ketoglutarate in the absence of NH_4Cl [see Krebs & Johnson, 1937, 1; Weil-Malherbe, 1937] competes with reaction (3). As the NH_3 concentration rises, the rate of (4) decreases owing to the conversion of α -ketoglutaric into α -iminoglutaric acid. Thus our method of calculation would yield too low values for the extra CO_2 .

As regards the glutamic acid formation in the absence of added NH_3 , this may be due to transamination or amination from endogenous amino-compounds or NH_3 .

α -Ketoglutaric acid and NH_3 in heart muscle

Similar experiments on heart muscle are recorded in Table III. The increases in succinic acid, glutamic acid and CO_2 formation after addition of NH_4Cl and α -ketoglutarate are distinct, but smaller than in kidney cortex. It is noteworthy that the succinic acid formation is considerably smaller than the glutamic acid formation in the experiment on sheep heart. This suggests that there are other reactions, in addition to (3), in which glutamic acid is synthesized.

Table III. α -Ketoglutaric acid and NH_3 in minced heart muscle

Incubation period 120 min.

Species	Quantity of tissue	Substrates added (final conc.)	$\mu\text{l. CO}_2$	$\mu\text{l. succinic acid}$	$\mu\text{l. glutamic acid}$
Sheep	500 mg. fresh muscle in 3.6 ml. 0.1 <i>M</i> phosphate buffer, <i>pH</i> 7.1	—	22	50	35
		0.02 <i>M</i> α -ketoglutarate	160	90	149
		0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	277	236	420
		0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl , 0.02 <i>M</i> citrate	270	139	402
		—	—	31	3
Pig	370 mg. fresh muscle in 3.4 ml. 0.1 <i>M</i> phosphate buffer, <i>pH</i> 7.1	0.02 <i>M</i> α -ketoglutarate	—	76	70
		0.02 <i>M</i> α -ketoglutarate, 0.006 <i>M</i> NH_4Cl	—	146	110
		—	—	—	—
		—	—	—	—

Table IV. Total CO_2 production in the presence of α -ketoglutarate and NH_4Cl in various tissues

Tissue	Substrates added (final conc.)	Q_{CO_2}
Guinea-pig kidney	0.02 <i>M</i> α -ketoglutarate	2.9
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	8.2
Guinea-pig kidney	—	2.04
	0.02 <i>M</i> α -ketoglutarate	3.07
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	7.9
	0.04 <i>M</i> α -ketoglutarate, 0.04 <i>M</i> NH_4Cl	9.4
	0.02 <i>M</i> pyruvate	4.0
	0.02 <i>M</i> pyruvate, 0.02 <i>M</i> NH_4Cl	4.6
	0.02 <i>M</i> oxaloacetate	7.5
Rat kidney	0.02 <i>M</i> oxaloacetate, 0.02 <i>M</i> NH_4Cl	7.9
	—	2.35
	0.02 <i>M</i> α -ketoglutarate	3.42
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	7.84
	0.02 <i>M</i> pyruvate	4.68
	0.02 <i>M</i> pyruvate, 0.02 <i>M</i> NH_4Cl	5.29
Rat liver	—	6.4
	0.02 <i>M</i> α -ketoglutarate	7.5
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	6.2
Pigeon liver	0.02 <i>M</i> α -ketoglutarate	4.30
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	5.85
Pigeon brain	0.02 <i>M</i> α -ketoglutarate	1.82
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	2.08
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> pyruvate	3.74
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> pyruvate, 0.02 <i>M</i> NH_4Cl	3.92
Guinea-pig brain	—	0.78
	0.04 <i>M</i> α -ketoglutarate	1.66
	0.04 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	1.52
Rat intestine	—	2.18
	0.02 <i>M</i> α -ketoglutarate	3.22
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	3.00
Rat testis	—	1.18
	0.02 <i>M</i> α -ketoglutarate	1.33
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	1.43
Rat spleen	—	1.98
	0.02 <i>M</i> α -ketoglutarate	2.09
	0.02 <i>M</i> α -ketoglutarate, 0.02 <i>M</i> NH_4Cl	1.80
Pigeon breast muscle	—	0.10
	0.02 <i>M</i> α -ketoglutarate	1.64
	0.02 <i>M</i> α -ketoglutarate, 0.04 <i>M</i> NH_4Cl	1.65
	0.02 <i>M</i> α -ketoglutarate, 0.04 <i>M</i> NH_4Cl , 0.02 <i>M</i> citrate	1.57

*Anaerobic CO₂ production in the presence of α -ketoglutaric acid
and NH₃ in various tissues*

Of the products of reaction (3), CO₂ is the easiest to determine. We have used the determination of the CO₂ production in the presence of α -ketoglutarate and NH₄Cl in order to test a series of tissues for reaction (3). Brain, intestine, spleen, liver, testis and pigeon breast muscle show no significant increase in CO₂ production (Table IV). Kidney and heart thus appear to be the only tissues in which reaction (3) is of major importance.

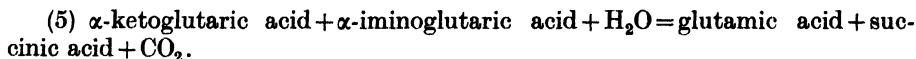
Effects of β -hydroxybutyrate and citrate on the synthesis of glutamic acid. In order to see whether reaction (2) occurs in surviving tissues a series of tissues (rat liver, rat and guinea-pig kidney cortex, guinea-pig, pigeon and sheep brain, sheep heart muscle and pigeon breast muscle) were incubated anaerobically with α -ketoglutarate, NH₄Cl and *dl*- β -hydroxybutyrate. After incubation, acetoacetic acid was determined by the aniline citrate method [Edson, 1935]. No significant amounts of acetoacetic acid were found and it must therefore be concluded that reaction (2) does not occur to an appreciable extent in the tissues tested, although the tissues appear to contain all the catalysts required for this reaction.

To test for reaction (1) citrate was added together with α -ketoglutarate and NH₄Cl to various tissues. Citrate rapidly forms isocitrate under the conditions of the experiment [Johnson, 1939] and if reaction (1) occurred an increased glutamic acid formation would be expected; no increase was found, however (Table III).

Reactions analogous to (3). Guinea-pig kidney cortex, liver and brain and pigeon breast muscle were incubated anaerobically with ammonium pyruvate and ammonium oxaloacetate, but no significant increase in CO₂ production was observed (see Table IV). There is therefore no reason to assume that other α -ketonic acids react in the same way as α -ketoglutaric acid.

DISCUSSION

α -Iminoglutaric acid. Knoop & Oesterlin [1925] found that solutions containing α -ketoglutaric acid and NH₃ yield glutamic acid on catalytic dehydrogenation. This fact may be taken as conclusive evidence of the existence of α -iminoglutaric acid in these solutions and reaction (3) may therefore be written in the following form:



The analogy between (5) and the simple dismutation of α -ketonic acids (4) is obvious. Reaction (5) may be considered as a special form of dismutation in which the α -imino-acid replaces one of the α -ketonic acids.

Glutamic acid as a hydrogen carrier. If reaction (3) is followed by the re-oxidation of glutamic to α -iminoglutaric acid, the system glutamic acid \rightleftharpoons iminoglutaric acid acts as a hydrogen-transporting system, a conception which was put forward some time ago, in a general way, by Knoop & Oesterlin [1925]. This would explain previous observations [Krebs, 1932; Edson, 1935] which showed that addition of NH₄Cl catalytically increases the respiration in kidney cortex in the presence of α -ketoglutarate and of those substances which may give rise to the formation of α -ketoglutarate, such as lactate, pyruvate or glucose [see Krebs & Johnson, 1937, 2]. The fact that the effect of NH₄Cl is

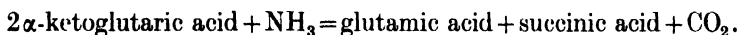
not observed in other tissues in which reaction (3) does not occur supports this explanation.

It is also suggestive to explain the widespread occurrence of glutamic dehydrogenase in animal tissues by its specific function as a hydrogen carrier in cellular respiration. In connexion with this hypothesis arises the question of the nature of the hydrogen donators for iminoglutaric acid. Our attempts to find donators other than α -ketoglutaric acid were so far without positive results. Glucose, lactate, pyruvate, α -glycerophosphate, *dl*-glyceric aldehyde had no effect on the reduction of iminoglutaric acid in sliced brain cortex.

Oxidative breakdown of α -ketoglutaric acid. Since reaction (3) does not occur in all the tissues which oxidize α -ketoglutaric acid there must be other mechanisms, e.g. in pigeon breast muscle, or in brain, whereby α -ketoglutaric acid is broken down. In kidney cortex, however, reaction (3) appears to be the chief pathway of the breakdown of α -ketoglutaric acid.

SUMMARY

The following reaction was found to take place when α -ketoglutarate and NH_4Cl were added to sliced kidney cortex or minced heart muscle:



This reaction is probably a step in the normal oxidative breakdown of α -ketoglutarate and of those substances which give rise to α -ketoglutarate. This is borne out by the fact that NH_4 salts catalytically increase the rate of oxidation of α -ketoglutarate.

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CCXXXIV. URICASE PURIFICATION AND PROPERTIES

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SINCE the work of Schittenhelm [1905], Wiechowsky & Wiener [1909], Batelli & Stern [1909; 1912] and others, it is well known that cells of different organisms contain an enzyme catalysing the oxidation of uric acid to allantoin. Batelli & Stern, who described this enzyme under the name of uricase, were the first to determine its fundamental properties and the mechanism of the reaction it catalyses. It was shown by this work that during the oxidation of one molecule of uric acid to allantoin, one atom of oxygen and one molecule of water are taken up while one molecule of CO_2 is given off, the R.Q. of the reaction being equal to 2. Other workers [Felix *et al.* 1929; Schuler, 1932] have found, however, that under certain conditions the O_2 uptake and the CO_2 -formation have different *pH*-optima. This result, according to Schuler, strongly supports the view that the reaction takes place in two steps: (1) oxidation of uric acid to an intermediate compound (oxy-acetylen-diurein-carboxylic acid) catalysed by uricase, and (2) decarboxylation of this compound which is either spontaneous or catalysed by another enzyme.

On the other hand, Keilin & Hartree [1936, 1] have been unable to find any departure from the theoretical R.Q. of 2 at different *pH* values.

As an oxidizing enzyme, uricase occupies an intermediate position between the typical dehydrogenases and the oxidases. Although it is reversibly poisoned by KCN it combines with the substrate and activates it, as shown by the inhibitory effect on the reaction produced by some derivatives of uric acid, which act as competitive inhibitors. On the other hand the substrate activated by this enzyme does not react with any known hydrogen acceptor except O_2 which is invariably reduced to H_2O_2 [Keilin & Hartree, 1936, 2].

All attempts to purify uricase have been without great success until quite recently when Davidson [1938, 1, 2] succeeded in obtaining a purified preparation with a Q_{O_2} about 600 times higher than that of the acetone-dried pig's liver used as the starting material. As this purified preparation was practically free from copper and haematin but contained between 0.15 and 0.20 % of iron, Davidson suggested the possibility that iron is a constituent of the enzyme.

In this paper I propose (1) to describe an improved method of purification of uricase, and (2) to examine the properties of the purified preparation [*vide also* Holmberg, 1939].

Method of preparation

(1) Fresh pig's liver (or liver stored for one day at $+4^\circ$) is minced in the Latapie mincer and treated twice with 4 parts of acetone. The acetone is sucked off on a large Büchner funnel, the residue is left to dry for a few hours on filter papers and overnight in a desiccator. The dry preparation is ground in a porcelain mill and passed through a fine sieve. The resulting fine powder is used for the extraction.

(2) The extraction is carried out according to Davidson's method. 200 g. of powder are first washed with 2 l. of cool *M*/10 phosphate buffer *pH* 7.3. The residue is centrifuged off and extracted for 20 min. at 40° with 4 l. *M*/10 alkaline borate buffer solution *pH* 10.

(3) The crude extract is precipitated with 25–50 g. of barium acetate, centrifuged and the precipitate discarded. The excess of barium is removed with an equivalent amount of ammonium sulphate.

(4) The first two steps of purification are also identical with those used by Davidson. The cooled extract is precipitated with an equal volume of saturated ammonium sulphate, the precipitate centrifuged off and redissolved in distilled water so that the concentration of ammonium sulphate becomes about 1/10 saturation. This solution, while mechanically stirred, is heated to 55–60° for 5 min., rapidly cooled and the resulting precipitate centrifuged off and discarded.

(5) The turbid solution is treated twice with 40 g. barium acetate (dissolved in small amounts of distilled water); the precipitates are centrifuged off each time and discarded.

(6) The solution is brought to pH 7 and adsorbed with 50 ml. (about 2 g. dry weight) alumina C-γ. The adsorbate is centrifuged off and discarded. The solution is filtered through a folded filter, giving a clear, pale yellow solution.

(7) The solution is again precipitated with an equal volume of saturated ammonium sulphate, which has previously been neutralized with NaOH. The resulting small precipitate is centrifuged down, redissolved in about 200 ml. distilled water and rapidly filtered. After standing for 1–2 hr. a fine white precipitate is formed. It is centrifuged down, washed several times with distilled water and suspended in 25–30 ml. of distilled water. This precipitate, which contains the uricase in a state which is only sparingly soluble in alkaline buffer solutions, will be referred to as “purified uricase”. The yield of this preparation is about 7 mg. per 200 g. of acetone powder and it contains 10–14 % of the uricase present in the original extract.

Determination of activity

The activity of the preparation is estimated from the velocity of O_2 uptake during the first 15 min. in presence of 2 mg. of lithium urate as measured in Barcroft differential manometers in air at pH 9 and 39°. The flask of the manometer receives the enzyme in 0.1 *M* borate buffer solution, while the substrate is introduced from a dangling cup. As the crude preparation of the enzyme always contains catalase, the activity of the purified enzyme can be compared with that of the crude enzyme only on addition to the former of a drop of pure catalase solution which decomposes the H_2O_2 formed during the reaction [Keilin & Hartree, 1936, 1]. Without catalase the activity of the purified enzyme seems to be about 30 % higher than in its presence (Fig. 1). That H_2O_2 accumulates during this catalytic oxidation is shown by a strong and instantaneous colour reaction obtained on addition of peroxidase and *p*-phenylenediamine to the reaction vessel even after the first 5 min. of uricase activity. If, on the other hand, peroxidase and *p*-phenylenediamine are added before the beginning

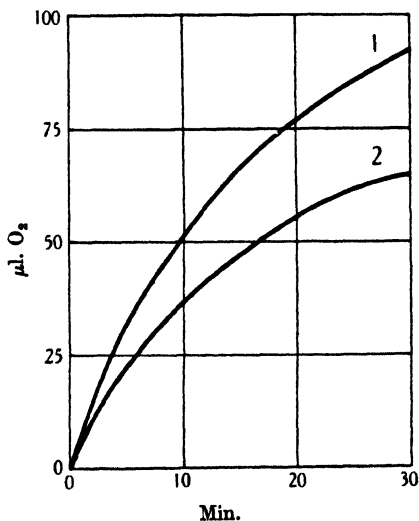


Fig. 1. Effect of catalase on O_2 uptake of 2 mg. uric acid and 30 μ g. uricase. Curve 1: uricase + uric acid. Curve 2: uricase + uric acid + catalase.

of the reaction, the colour is only gradually developed [Keilin & Hartree, 1936, 1].

The accumulation of H_2O_2 can also be demonstrated by the addition of catalase from a dangling tube at the end of an experiment. The rapid decomposition of H_2O_2 which takes place immediately after mixing the solution with catalase produces a positive pressure which can be read on the manometer.

The enzyme concentration used in these experiments was so adjusted that the velocity of the reaction did not exceed $60\mu\text{l.}$ per 15 min. The activity of the purified uricase preparation estimated in this way and expressed as Q_{O_2} is approximately 6000. This preparation is therefore 1200 times more active than the acetone-dried powder of liver, the Q_{O_2} of which is approximately 5.

Properties of purified uricase

The purified uricase preparation is insoluble in water and forms a very fine precipitate which settles very easily. It is insoluble in neutral and slightly alkaline buffers, and only sparingly soluble at pH 10. In the dried state it has a pale brownish-green colour. The preparation gives a negative reaction for carbohydrate. The nitrogen content of a sample, which was not completely free from inorganic contamination with barium phosphate, was about 13.5 %. The iron content of the same sample was 0.025 %. The suspensions and alkaline solutions of such preparations containing about 5 mg. dry weight per ml. are colourless and show no absorption bands in the visible region of the spectrum. Only when treated with pyridine and reducer do they reveal the very faint α -band of a protohaemochromogen.

The preparation can be kept as a suspension in distilled water in the cold for at least one week without an appreciable loss of activity.

Effect of inhibitors

Purified uricase is very sensitive to cyanide, the presence of 10^{-4} M KCN in the dangling cup being sufficient to abolish its activity. This inhibitory effect, as in the case of a crude enzyme preparation [Keilin & Hartree, 1936, 1] is perfectly reversible.

Sodium azide produces only a very slight inhibition at pH 7, but this effect lasts only for the first 5 min. of the reaction and then vanishes.

Sodium sulphide, $\alpha\alpha$ -dipyridyl, dithizone and diethyldithiocarbamate have no inhibitory effects. Among metals copper exerts a very strong poisoning effect, 2×10^{-5} M copper sulphate giving 75 % inhibition using $60\mu\text{g.}$ of enzyme. Iron is much less effective, 10^{-3} M ferrous sulphate inhibits the same amount of enzyme by only 35 %.

Manganese inhibits the enzyme in concentration as low as 10^{-4} M but this effect only develops gradually.

H_2O_2 , as in the case of other enzymes, has a marked inhibitory effect which can, however, be completely abolished by the presence of 0.2 % of gelatin. Very small amounts of diethyldithiocarbamate also abolish the inhibition by H_2O_2 . It seems probable, therefore, that this inhibition is due to the presence of traces of copper (Fig. 2).

Formation of CO_2 in the oxidation of uric acid by purified uricase

It was suggested previously [Schuler, 1932] that the oxidation of uric acid to allantoin is a two-step reaction with the formation of an intermediate compound undergoing decarboxylation either spontaneously or through co-operation with another enzyme.

The study of the R.Q. of this reaction catalysed by a highly purified uricase does not, however, support the view as to the existence of a decarboxylating enzyme working in co-operation with uricase. In fact, the R.Q. of the reaction

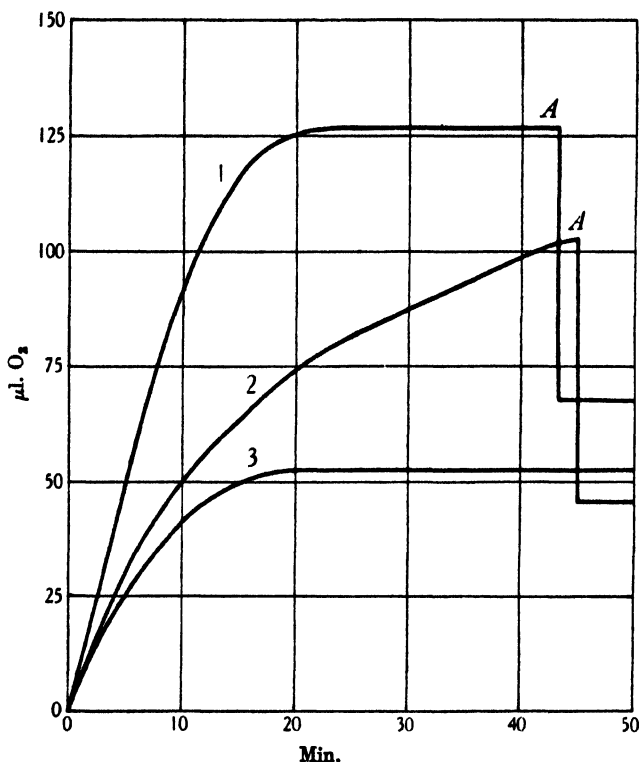


Fig. 2. Experiment showing poisoning action on uricase of accumulated H_2O_2 , and protection by diethyldithiocarbamate. Uric acid 1 mg. + uricase 35 μg . The amount of H_2O_2 accumulated is shown by the increase in pressure after adding catalase. Curve 1: uricase + uric acid + 20 diethyldithiocarbamate. Curve 2: uricase + uric acid. Curve 3: uricase + uric acid + catalase. At A catalase added from dangling cup.

measured at pH 7.3 in presence and in absence of catalase are 1 and 2 respectively. On the other hand, it seems very unlikely that the complicated procedures of the purification could lead to a preparation containing two entirely distinct enzymes.

Coupled oxidations

It is well established now that the H_2O_2 formed in the primary oxidation reaction can be utilized for secondary or coupled oxidation. In the coupled oxidation H_2O_2 is activated either by metals, haematin or peroxidase present in or added to the preparation [Thurlow, 1925; Harrison & Thurlow, 1926]. It was thus possible with H_2O_2 , formed in the primary oxidation catalysed by purified uricase, to oxidize substances like sodium sulphide, hydroxylamine, *p*-aminophenol and *p*-phenylenediamine.

An entirely different type of secondary or coupled oxidation reaction is that of alcohol. It was shown by Keilin & Hartree [1936, 2] that alcohol added to the crude uricase and uric acid doubles the O_2 uptake by this system. They have also

shown that alcohol produces a similar effect on xanthine oxidase and hypoxanthine, only if catalase is added to the system. In both cases, however, alcohol undergoes oxidation to acetaldehyde. They have naturally concluded that in crude uricase preparation, the oxidation of alcohol by H_2O_2 formed in the primary reaction was also catalysed by catalase present in the preparation. That this is so can be clearly demonstrated now with the purified uricase preparation which is completely free from catalase. As is shown in Fig. 3 the addition of

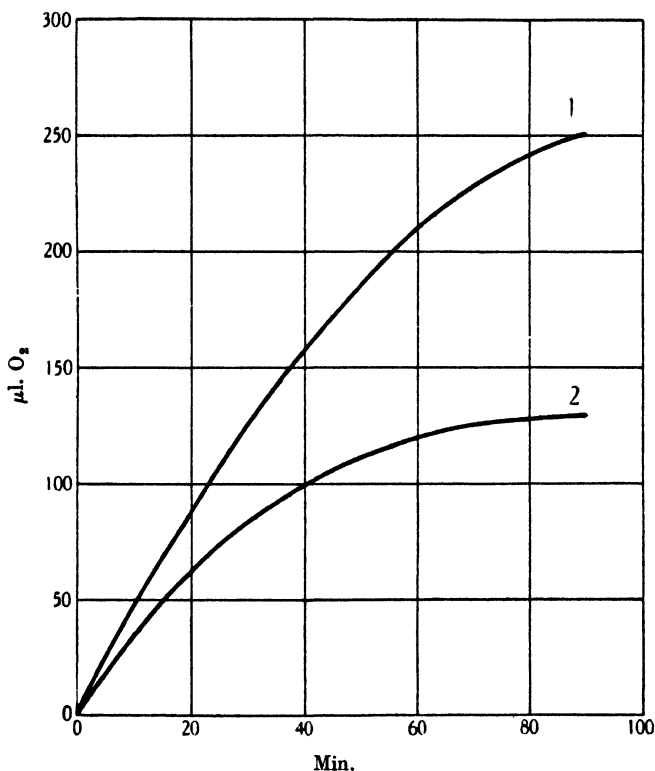


Fig. 3. Effect of 12 mg. ethyl alcohol on O_2 uptake by 2 mg. uric acid + 30 μg . uricase + one drop strong catalase. Curve 1: uricase + uric acid + catalase + alcohol. Curve 2: uricase + uric acid + catalase. The same amount of alcohol has no effect, on the O_2 uptake by uricase + uric acid without catalase.

alcohol to the purified uricase-uric acid system has no effect on this system while in presence of catalase it doubles the O_2 uptake and undergoes oxidation to aldehyde.

Nature of uricase

The fact that cyanide reversibly inhibits the catalytic action of uricase makes it very probable that this enzyme contains a heavy metal in its active group.

Although the amount of iron in these purified preparations is very small (0.02 %) the possibility that uricase contains iron cannot be definitely ruled out. In fact, as we have no indication yet as to the degree of purity of these preparations, and assuming that they are only 10–20 % pure, it is still conceivable that the iron or even the haematin which is just detectable, might be associated with the enzyme.

SUMMARY

A modification of the method described by Davidson is given whereby it has been possible to obtain uricase with a Q_{O_2} of about 6000. The preparation has a very low iron content (about 0.025 %). The purified preparation has the same R.Q. as crude preparations and it is, therefore, considered improbable that the oxidation and decarboxylation of uric acid are catalysed by different enzymes.

An account is given of the action of different inhibitors on the purified uricase preparation, and of some coupled oxidations that can be produced by the H_2O_2 formed in the reaction are described.

I wish to express my deep gratitude to Prof. D. Keilin for giving me facilities to work in his laboratory and for all the help he has given me with this work, and also to Dr T. Mann for much help and advice.

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ADDENDUM

Note added 1 October 1939. A purified uricase preparation analysed by the dithizone method for lead and zinc gave the following results:

A 6 mg. sample was found to be free from lead, while another 10 mg. sample was found to contain 13 μ g. zinc; i.e. 0.13%. Considering that liver, the source of uricase preparations, is rich in zinc no definite conclusion can be drawn from this single estimation. It suggests nevertheless that further study of the relationship between uricase and zinc carried out on a larger scale will be of interest.

CCXXXV. THE SPLITTING OF HAEMOGLOBIN BY ACIDS

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(Received 14 September 1939)

It is well known that haemoglobin is split by acids into its protein constituent, globin, and the prosthetic group haem. That these two dissociation products can be recombined has long been known, and the resynthesis has been extensively studied. The purpose of the present work was to investigate the products of fission and reconstitution with the aid of the ultracentrifuge. Globin has been previously examined in the ultracentrifuge [Svedberg, 1930] and found to be heterogeneous as regards molecular weight. Svedberg suggested that this heterogeneity was caused by severe chemical conditions during preparation of the globin, and, further, that milder methods would probably yield a monodisperse product. Some of the results of the present investigation have already been quoted by Svedberg [1937; 1938].

EXPERIMENTAL

The method of Anson & Mirsky [1930] was used for the splitting and reconstitution of haemoglobin. Horse COHb was treated with 0.1 *N* HCl, and the globin thus split off was precipitated by acetone, separated, air-dried and dissolved in water. The solution obtained was slowly neutralized with 0.1 *N* NaOH, which precipitated denatured protein, and the "native" globin was further freed from denatured globin (paraglobin, according to Roche [1930]) by 40 % saturation with ammonium sulphate. The native globin was then precipitated by adding solid ammonium sulphate (16 g./100 ml. solution), redissolved and dialysed against distilled water in the cold. The solutions obtained were never very stable. (Cf. Roche *et al.* [1932], who report a similar instability of native horse globin.) During dialysis part of the globin was always precipitated; moreover, a clear solution of globin soon became turbid if kept at room temperature. On electro-dialysis the protein was completely precipitated.

Alkaline haem solutions were prepared by adding to the acetone solution 1 % of its volume of 2 *N* sodium acetate, and dissolving the precipitate so obtained in a buffer consisting of equal parts of Na_2CO_3 and NaHCO_3 [cf. Anson & Mirsky, 1930]. By adding this solution to a solution of neutral globin, methaemoglobin was obtained; this was reduced by the addition of a small amount of $\text{Na}_2\text{S}_2\text{O}_4$, then immediately saturated with CO and dialysed against distilled water. Some precipitate always formed during the coupling and subsequent dialysis, but the residual solution was very stable and could be kept for months at 4°. Electro-dialysis, however, precipitated the protein. In order to obtain a successful coupling of haem and globin, it was necessary to use a freshly prepared solution of haem [cf. Herzog, 1933].

Sedimentation experiments. Sedimentation experiments were carried out in the ultracentrifuge at a speed of 59,000 r.p.m., corresponding to a force of gravity $\times 250,000$. The temperature varied from 25 to 30°. Observations were

made with the light absorption method and with the scale method of Lamm [1937], both of which have been described by Svedberg [1937; 1938] and by Svedberg & Pedersen [1939]. The acid acetone globin, namely, an aqueous solution of the globin precipitated by acetone from the HCl solution, examined before neutralization and dialysis, appeared to be heterogeneous with regard to molecular weight. The size of molecules present varied within a wide range around a maximum corresponding to a sedimentation constant (reduced to water at 20°, s_{20}) of about 2.5.¹ In order to decide whether this heterogeneity was caused by the acetone precipitation, experiments were also made with haemoglobin in acid solution (pH about 2), where fission into haem and globin will have taken place. The haem was observed by light absorption at 546m μ and was found to sediment at a rather rapid rate (s_{20} = 7.5), showing that it forms aggregates in the solution. The globin could be observed by light absorption in the ultra-violet (chlorine and bromine filters) and also by the scale method. It sedimented more slowly than the haem and was appreciably heterogeneous. The sedimentation constant by either method was found to be 2.2, which is in satisfactory agreement with that obtained for the acetone-precipitated globin, if allowance is made for the unsatisfactory sedimentation diagrams obtained with heterogeneous substances.

The neutralized globin solutions, from which paraglobin had been removed, were dialysed against phosphate buffers at pH 7.0 (in general 0.2 M NaCl + 0.03 M Na₂HPO₄ + 0.02 M KH₂PO₄) before ultracentrifuging. This material gave much better sedimentation curves than the acid acetone globin, but no preparation was found to be entirely homogeneous. The best criterion of homogeneity is that the diffusion constant, calculated from a sedimentation experiment, should agree with the results from a static diffusion experiment. The diffusion constant of globin is 6.5 (see below). In the best sedimentation experiment the values 7.8, 8.1 and 9.8 were obtained for the diffusion constant at different times. This is a fair agreement, but it still indicates a slight inhomogeneity. The preparation used for this experiment was carried out entirely at 4°, which proved to be more suitable than room temperature. The sedimentation constants were readily reproducible. The results obtained from four different preparations are recorded in Table I.

Table I

Exp. no.	Temperature of neutralization of globin °C.	Method of observation	Sedimentation constant	Protein concentration %
H 10	+20	Light absorption	2.64	—
G 6	+20	Scale	2.59	0.2
G 10	+ 4	"	2.53	0.2
G 12	+ 4	"	2.51	1.0

The protein concentrations are calculated by integration of the sedimentation curves, assuming a refractive index increment of 1.96×10^{-3} for globin at a wave-length of 436m μ . Owing to the instability of the solutions, it was difficult to determine the concentration by any other method. The figures given are to be regarded as approximate. In concentrations below 1%, the sedimentation constant is independent of concentration.

The synthetic COHb proved to be homogeneous in the ultracentrifuge. Observations were made by the light absorption method at a wave-length of 546m μ , and the photometer curves obtained indicate monodispersity as for

¹ Sedimentation constants are given in units of 10^{-13} .

native haemoglobin. The sedimentation constant was about 4.5, in a pH range from about 6 to 9.8, for moderate protein concentrations (0.5–1 %) and in buffer solutions of a molarity of about 0.2. Exposures at 546m μ and with ultra-violet light made during the same experiment gave the same sedimentation constant, and the ultra-violet photographs show that no protein is left behind the sedimenting boundary. This provides good evidence that all the globin present after dialysis has recombined with haem, and that denatured globin has been precipitated. At a low concentration (0.1 %) of the synthetic haemoglobin, s_{20} was 4.0, and the same value was obtained from an experiment at a relatively high concentration (2.8 %), the pH being 6.9 in each case. The pH stability diagram of the synthetic COHb is shown in Fig. 1; it is almost identical with that of native haemoglobin [Andersson & Pederson, unpublished].

As mentioned above, it was observed that the haem in acid solution sediments rather rapidly. In alkaline solutions the same effect was shown though to a lesser degree (in different experiments s_{20} varied from 2.2 to 3.6). In all instances the aggregates formed were very polydisperse. Aggregation in alkaline solutions was noted by Anson & Mirsky [1930] who observed that the haem did not pass through collodion membranes made to retain haemoglobin.

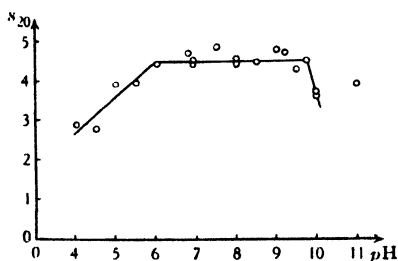


Fig. 1.

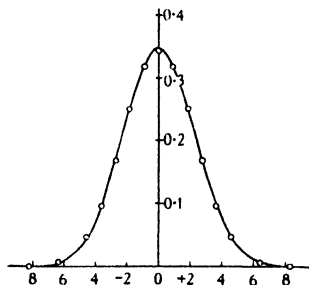


Fig. 2.

Fig. 1. pH-stability diagram of synthetic carbon monoxide haemoglobin.

Fig. 2. Diffusion curve of native globin. Abscissa: distance from original boundary position (mm.). Ordinate: scale line displacement (mm.). Time after start: 36,000 sec. The experimental curve is continuous, the circles indicate the theoretical curve for a homogeneous substance.

Diffusion experiments. The best globin preparation was used for a diffusion experiment at 20°, using Lamm's method [1937]. The value $D_{20} = 6.5^1$ was obtained (concentration 0.54 %). The diffusion curves obtained showed a very slight indication of heterogeneity (see Fig. 2, which gives the globin diffusion curve, compared with a normal distribution curve that should be given by a homogeneous substance).

A 0.8 % solution of the synthetic haemoglobin gave a diffusion constant of 6.3, which is in good agreement with the value (6.27 in a 0.8 % solution) found by Tiselius & Gross [1934] for native horse haemoglobin. Polson [1939, 1] has reported a higher value for the diffusion constant of horse haemoglobin (7.09 in a 1 % solution).

Molecular weight calculations. The molecular weights of the proteins investigated have been calculated by the formula

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

¹ Diffusion constants are given in units of 10⁻⁷.

[Svedberg, 1937; 1938; 1939]. The partial specific volume, V , for COHb was shown by Svedberg & Fåhræus [1926], to be 0.749. The same value has been used here for the synthetic haemoglobin and for the native globin, which presumably cannot introduce any large error. By this means a molecular weight of 37,000 has been obtained for globin and 69,000 for synthetic haemoglobin. The frictional ratio, f/f_0 , namely, the ratio between the observed frictional constant and that of a compact spherical molecule of the same weight [Svedberg, 1939] was 1.47 for globin and 1.23 for synthetic haemoglobin.

Electrophoretic experiments. The synthetic COHb was investigated in the electrophoresis apparatus with quartz tubes, described by Tiselius [1930, cf. Pedersen, 1933]. All the protein solutions used for electrophoresis were dialysed overnight at room temperature against buffers of ionic strength 0.02. The electrophoresis experiments were carried out at 20°. Control experiments were made with native COHb which had been treated with a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ and then dialysed. The controls gave results which are in good agreement with those obtained by Pedersen, who found an isoelectric point of 6.92 for horse haemoglobin, using the same apparatus (unpublished results, quoted by Svedberg [1939]). It is therefore evident that the hydrosulphite has not affected the protein.

Three different preparations of synthetic haemoglobin were examined. The results are given in Fig. 3. All the preparations were electrophoretically homogeneous, and all showed the same picture in the ultracentrifuge. On electrophoresis, one of them (III) gave results that agree with those obtained from the native protein (isoelectric point 6.92), but the two others (I and II) had a slightly more acid isoelectric point (6.72), and the whole mobility curve was moved about 0.2 pH unit to the acid side. The difference is definitely outside the range of experimental error.

Light absorption experiments. The light absorption of native and recoupled COHb was measured in a König-Martens spectrum photometer. The maxima of the characteristic absorption bands were found to be at 539 and 568 $m\mu$ for the native protein, and 537 and 567 $m\mu$ for the recoupled haemoglobin. The differences are not necessarily significant, as the limits of error of the instrument are rather wide. The extinction coefficients, relative to the nitrogen contents of the solutions (Kjeldahl), agree well for the two preparations. This fact shows that the molecules contain the same number of haem groups per equivalent of globin. These results are in agreement with those reported by Herzog [1933].

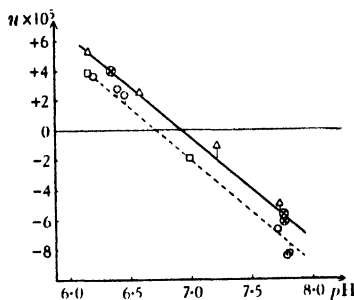


Fig. 3. Electrophoretic mobility (u), of native and synthetic carbon monoxide haemoglobin. The continuous curve is the mobility curve of native Hb and synthetic Hb III; the dotted curve refers to synthetic Hb I and II. \odot Native Hb. \square Synthetic Hb, I. \circ Synthetic Hb, II. \triangle Synthetic Hb, III.

DISCUSSION

Roche & Combette [1937] have measured the molecular weight of synthetic haemoglobin by the osmotic pressure method. They obtained a value of 66,000 for the methaemoglobin formed, in good agreement with their values for methaemoglobin prepared directly from oxyhaemoglobin. During the present study methaemoglobin formed by coupling haem and globin was found to have a

sedimentation constant of about 4.5 (three different preparations gave values of 4.55, 4.42, and 4.45), which corresponds closely with the results obtained by Roche & Combette. In this work, COHb was used in most experiments, because it appears to be more homogeneous than methaemoglobin, as shown by Pedersen for native haemoglobin (personal communication), and indicated also by the experiments on recombined haemoglobin. The experiments on synthetic COHb show, moreover, that the molecular weight of the recombined protein is the same as that of the native protein (69,000 from latest ultracentrifugal measurements, [cf. Svedberg & Fåhræus, 1926; Svedberg, 1939]. The frictional ratios before fission and after recombination are also the same, which would indicate that the shape and the hydration of the molecule are unchanged.

The electrophoretic measurements are possibly more sensitive to changes in the linkage of haem and globin, and would seem, at least in some instances, to give reason for doubt as to the identity of the native and the resynthesized haemoglobins. This view is further supported by the fact that electro dialysis causes precipitation of the recombined protein and not of the native protein.

Roche *et al.* [1932] measured the osmotic pressure of solutions of native ox globin, and found that the molecular weight depended on the protein concentration. Their experiments extended over a wide concentration range, from 0.8 to 10.18 g./100 ml. They also investigated native horse globin at pH 5.6, but only at low concentrations (< 1.11%). In solutions of about 1% they report a molecular weight for native globin of about one-half that of haemoglobin. The present results support this finding. The molecular weight of native horse globin is independent of concentration below 1%, which is not contradictory to the results of Roche *et al.* for higher concentrations. It is therefore probable that the splitting of haemoglobin into haem and globin by acid is accompanied by a splitting of the protein molecule into halves. Steinhart [1938] has shown that the haemoglobin molecule can be split into halves by urea. Pedersen & Andersson found that haemoglobin is split into halves in very dilute solutions (unpublished work, quoted by Svedberg [1939]). It thus seems evident that the linkages between the two halves of the haemoglobin molecule are rather weak.

It was not possible to obtain globin in as homogeneous a state as native or recoupled haemoglobin. This would indicate that the globin solutions always contained some denatured protein still in solution, or else that part of the globin was present as complexes which broke up when the haem was combined with the globin. The fact that some precipitate was always formed on recoupling gives support to the former view, and the precipitate given was probably a combination product of haem and denatured globin.

The frictional ratio of haemoglobin is 1.23 and that of globin 1.47. This would indicate that the smaller molecule has a more extended shape. As pointed out by Polson [1939, 2], the products of fission of protein molecules have often higher frictional ratios than the original molecule. Polson found that the pH dissociation of protein takes place along the long axis of the molecule. Even if his numerical calculations are not strictly accurate (as he does not take into account the hydration of the molecules) it is probable that his finding is applicable to the splitting of haemoglobin by acid.

SUMMARY

1. Globin and haem, obtained by splitting horse haemoglobin with acid, could be recoupled to form a haemoglobin that has the same molecular weight (69,000) as native haemoglobin, the same pH-stability diagram and the same molecular shape.

2. Electrophoretic experiments have, in some instances, given reason to doubt the identity of native and recoupled haemoglobins.

3. Native globin has a molecular weight about half that of haemoglobin. It is probable from the frictional ratios of the molecules that the splitting has taken place along the long axis of the haemoglobin molecule.

The author wishes to thank Prof. T. Svedberg for suggesting this research, for the facilities provided at his laboratory and for his kind interest in the work. Thanks are due also to Dr K. O. Pedersen for valuable advice and to Dr G. S. Adair and Mrs M. E. Adair for useful criticism.

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CCXXXVI. EXPERIMENTS ON AMINO-ACIDS

I. THE PARTITION OF ACETAMINO-ACIDS BETWEEN IMMISCIBLE SOLVENTS

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(Received 5 October 1939)

THE work described in this series of papers was undertaken with a view to extending the methods available for the isolation of amino-acids from protein hydrolysates. At this stage of the work it seems desirable to publish what has already been done, so that others may make use of the observations.

Neuberger [1938, 1] observed that after acetylation by ketene of an enzymic digest of egg albumin, followed by acidification, a proportion of the total N was rendered extractable from aqueous solution into chloroform. From this, it seemed possible that acetamino-acids might have widely differing partition coefficients between chloroform and water, and that this might afford the basis of a general method for the isolation of amino-acids from protein hydrolysates. With Dr Neuberger's agreement a preliminary investigation of the partition coefficients of a number of acetamino-acids was therefore undertaken. The results of this are summarized in the present paper.

The partition coefficients of the *N*-acetyl derivatives of the naturally occurring amino-acids between chloroform and water were found to cover a very wide range. Some ether-water and ethyl acetate-water systems were also investigated. The figures obtained with ether did not differ very strikingly from those obtained with chloroform. A selection of the ethyl acetate figures is given below.

The experimental technique used for determining the partition coefficients was to shake together in a separating funnel known volumes of chloroform and an aqueous solution of the solute under investigation, and then to estimate the amount of solute in each phase either by titration against standard alkali or by N determination. In some cases, however, where the coefficient was too high for such direct determination, a rough estimate could be made by subjecting an aqueous solution of the compound to extraction by a known volume of chloroform in the continuous liquid-liquid extractor described by Neuberger [1938, 1]. The amount of chloroform passing through the aqueous layer in this apparatus can be readily determined with reasonable accuracy by measuring the temperature rise and rate of flow of the condenser water from time to time. The Neuberger extractor shows a complete absence of entrainment; a saturated aqueous solution of Na_2SO_4 was extracted in the apparatus with 50 vol. of chloroform, and on evaporating the chloroform extract to dryness it was impossible to detect SO_4^{2-} in the residue.

By using a substance of known partition between chloroform and water, and by determining its rate of disappearance from the aqueous layer in the apparatus after extraction by different volumes of chloroform, it was found that the "plate efficiency" of the Neuberger extractor (aqueous layer 500 ml., passing 1.0–1.5 l. of chloroform per hr.) was 70–80 %.

The preparation and properties of the compounds used are described in an appendix to this paper.

In the following tables:

$$P = \frac{\text{concentration of solute in aqueous phase}}{\text{concentration of solute in organic solvent phase}},$$

c = concentration of solute in aqueous phase, expressed as mg. per ml.

Effect of concentration on P

This is not very marked, and P tends to reach a constant value at high dilutions. In general P does not vary by more than a factor of 2 at concentrations below 10 mg. per ml. in the aqueous phase. Fig. 1 illustrates the variation

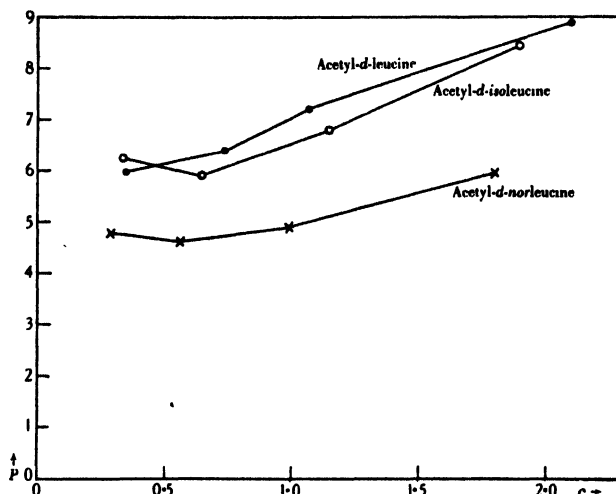


Fig. 1. Partition isotherms of acetyl-*d*-leucine, acetyl-*d*-isoleucine and acetyl-*d*-norleucine between chloroform and water phases at 37°. (P and c have same significance as in text.)

of P against c for acetyl-*d*-isoleucine, acetyl-*d*-norleucine, and acetyl-*d*-leucine at 37°. No attempt has been made to give a theoretical treatment of these isotherms in terms of ionic dissociation or of association in the non-polar solvent.

Effect of temperature on P

P has in every case a considerable negative temperature coefficient. This is illustrated by the figures for the partition of acetyl-*d*-leucine given in Table I.

Table I. *Effect of temperature on partition of acetyl-d-leucine between water and chloroform*

Temp. °C.	P	c
4	13.4	0.6
24	8.8	0.5
37	6.0	0.4

Effect of salt on P

Raising the concentration of neutral salt in the aqueous phase leads invariably to a lowering of P for any given value of c . This "salting-out" effect is particularly noticeable with *N*-acetyl-*L*-tyrosine in the presence of Na_2SO_4 .

Table II. *Approximate partition coefficients of acetamino-acids between chloroform and water*

Compound	Temp. °C.	<i>P</i>	<i>c</i>
<i>ON</i> -Diacetyl- <i>dl</i> -tyrosine	18	7	8
Acetyl- <i>L</i> -phenylalanine	37	5	1
Acetyl- <i>d</i> -norleucine	37	5	1
Acetyl- <i>d</i> -isoleucine	37	6	1
Acetyl- <i>d</i> -leucine	37	7	1
Acetyl- <i>L</i> -proline	37	15	2
Acetyl- <i>L</i> -methionine	37	22	2
Acetyl- <i>L</i> -valine	37	30	2
Acetyl- <i>dl</i> - α -aminobutyric acid	37	40	2
Acetyl- <i>dl</i> -alanine	37	160	2
Acetyl-glycine	18	600	7
<i>N</i> -Acetyl- <i>L</i> -tyrosine	37	600	4
Diacetyl- <i>dl</i> -lysine	20	800	10 See Note 1
Acetyl- <i>L</i> -glutamic acid	20	>1000	6
<i>N</i> -Acetyl- <i>dl</i> -serine	20	>1000	—
<i>N</i> -Acetyl- <i>L</i> -hydroxyproline	20	>1000	—
<i>NN'</i> -Diacetyl- <i>L</i> -cystine	20	>1000	10 See Note 2

Note 1. Diacetyl-*dl*-lysine could not be prepared crystalline. (Neuberger [1938, 2] also reports failure to obtain a crystalline product on treating *L*-lysine with ketene and NaOH in aqueous solution.) For this determination, therefore, synthetic *dl*-lysine dihydrochloride was acetylated in aqueous solution by the method of du Vigneaud & Meyer [1932], with acetic anhydride and NaOH. A Van Slyke amino-N determination on an aliquot of the resulting solution (30 min. shaking) showed that 88% of the original —NH_2 groups were acetylated, corresponding to the conversion of at least 76% of the original lysine residues into diacetyllysine. *N* corresponding to 2% of this diacetyllysine was extracted by the passage through the Neuberger apparatus of 17 vol. of chloroform after acidification of the aqueous phase. The figure $P=800$ given above is probably lower than the true figure for diacetyllysine in pure aqueous solution, owing to the considerable amount of salt present in the acetylation mixture after acidification.

Note 2. *NN'*-diacetyl-*L*-cystine does not crystallize [Hollander & du Vigneaud, 1931]. It was accordingly prepared in solution by acetylation of *L*-cystine, and P was determined in the same way as for diacetyllysine (see note 1). This value of P is probably low, as a result of "salting-out".

Table III. *Partition of acetamino-acids between ethyl acetate and water phases*

Compound	Temp. °C.	<i>P</i>	<i>c</i>
Acetyl-glycine	18	36	3
Acetyl- <i>L</i> -glutamic acid	37	23	10
Acetyl- <i>dl</i> -alanine	18	18	3
Acetyl- <i>dl</i> - α -aminobutyric acid	18	7	4
<i>N</i> -Acetyl- <i>L</i> -tyrosine	37	1.4	8

It will be seen by comparison of the above tables that the ethyl acetate figures cover a much less wide range than the chloroform figures, also that the positions of *N*-acetyltyrosine and acetylglutamic acid are entirely different in the two series.

α -*N*-Acetylarginine and α -acetylhistidine contain free basic groupings, and are therefore unlikely to be extractable from water into chloroform. Evidence is presented in Paper II of this series that for α -acetylarginine this is the case.

APPENDIX

The preparation and properties of acetamino-acids

In preparing acetamino-acids from the corresponding free amino-acids, the acetylation was carried out, unless otherwise stated, with acetic anhydride and NaOH in aqueous solution at 0° [cf. Bergmann & Zervas, 1928; Chattaway, 1931; du Vigneaud & Meyer, 1932]. This method has been shown by du Vigneaud & Meyer [1932] to cause very little racemization. The molecular proportions used by these authors were employed in each preparation, unless otherwise stated, and the yields obtained were uniformly good. After adding H_2SO_4 exactly equivalent to the NaOH employed in the preparation, the product was separated from the resulting Na_2SO_4 by extraction with hot alcohol. After evaporation of the alcohol *in vacuo*, the product was recrystallized from a suitable solvent, as described below. The acid equivalent weights of the compounds were determined by titration against a standard NaOH solution, using phenol red as indicator. Compounds described here for the first time are indicated with an asterisk.

Acetyl-dl-alanine. Prepared from *dl*-alanine (B.D.H.). Recrystallized from acetone. m.p. 136°. Fischer [1903] gives m.p. 137°.

**Acetyl-dl- α -aminobutyric acid*. Prepared from *dl*- α -aminobutyric acid (B.D.H.). Recrystallized from ethyl acetate. m.p. 129–131°. (Found: C, 48.2; H, 7.20; N (Dumas), 8.9%. $\text{C}_6\text{H}_{11}\text{O}_5\text{N}$ requires C, 49.6; H, 7.58; N, 9.6%. Acid equiv. wt.: found 148; calc. 145.)

NN'-*Diacetyl-l-cystine*. See Note 2, Table II.

Acetyl-l-glutamic acid. Prepared from *l*(+)-glutamic acid [cf. Bergmann & Zervas, 1928; du Vigneaud & Meyer, 1932].

Acetyl-glycine. Prepared from glycine (B.D.H.) [cf. Chattaway, 1931].

**Acetyl-l-hydroxyproline*. The preparation and properties of this compound are described in Paper III of this series.

**Acetyl-d-isoleucine*. *dl*-isoLeucine (prepared from *sec*-butylmalonic ester) was resolved by the method of Locquin [1907]. The resulting *d*(-)-isoleucine had $[\alpha]_D^{21}$ -11.5° (water, *l*=2, *c*=2.7). This on acetylation yielded a product which was recrystallized from water. m.p. 150–151°. $[\alpha]_D^{20}$ -15.6° (alcohol, *l*=2, *c*=2.3). (Found: C, 55.5; H, 8.60; N (Kjeldahl), 8.4%. $\text{C}_8\text{H}_{15}\text{O}_5\text{N}$ requires C, 55.5; H, 8.67; N, 8.1%. Acid equiv. wt.: found 173; calc. 173.)

**Acetyl-d-leucine*. *dl*-Leucine (prepared from isobutylmalonic ester) was resolved for me by Mr S. Williamson, to whom I express my thanks. He used the method of Fischer & Warburg [1905]. The resulting *d*(+)-leucine was acetylated, and yielded a product which was recrystallized from water. m.p. 186–188°. $[\alpha]_D^{22}$ +23.2° (alcohol, *l*=2, *c*=3.7). (Found: C, 55.8; H, 8.76; N (Kjeldahl), 7.8%. $\text{C}_8\text{H}_{15}\text{O}_5\text{N}$ requires C, 55.5; H, 8.67; N, 8.1%. Acid equiv. wt.: found 175; calc. 173.)

Cherbuliez *et al.* [1930] describe the isolation of acetyl-*l*-leucine from a protein by distillation of its ethyl ester. Their product had m.p. 181° and $[\alpha]_D^{20}$ -16.99°. They remarked that their product had higher rotation and melting point than that of Karrer *et al.* [1921]. It will be noted that the present compound, of synthetic origin, has still higher m.p. and rotation than that of its optical antipode as isolated by Cherbuliez *et al.*

Diacetyl-dl-lysine. See Note 1, Table II.

Acetyl-l-methionine. Prepared from *l*-methionine isolated from an enzymic protein digest by Mr N. W. Pirie, to whom I express my thanks for the gift [cf. du Vigneaud & Meyer, 1932].

**Acetyl-d-norleucine*. *dl*-norLeucine (prepared from *n*-hexoic acid by bromination to α -bromo-*n*-hexoic acid, followed by amination) was resolved by the method of Marko [1908]. The resulting *d*(-)-norleucine had $[\alpha]_D^{22}$ -22.9° (18% HCl, *l*=0.5, *c*=5).

This on acetylation yielded a product which crystallized slowly from water. The compound is much more soluble in water than the corresponding derivatives of isoleucine and leucine, and might therefore be of use as an intermediate in the study of leucine fractions of protein origin.

The acetylated product had m.p. 112–114° and $[\alpha]_D^{25} - 0.2^\circ$ (alcohol, $l=2$, $c=2.4$). This very low rotation is not due to racemization, since the compound on acid hydrolysis shows the correct rotation for *d*(-)-*nor*leucine in acid solution. (Found: C, 55.9; H, 8.81; N (Kjeldahl), 8.0%. $C_9H_{15}O_3N$ requires C, 55.5; H, 8.67; N, 8.1%. Acid equiv. wt.: found 172; calc. 173.)

Acetyl-l-phenylalanine. Prepared from *l*(-)-phenylalanine (Hofmann-La Roche) [cf. du Vigneaud & Meyer, 1932]. The present product had m.p. 170–172°.

Acetyl-l-proline. Prepared from *l*-proline (isolated from a gelatin hydrolysate) [cf. Bergmann, 1935; du Vigneaud & Meyer, 1932].

**N-Acetyl-dl-serine*. The preparation and properties of this compound are described in Paper III of this series.

N-Acetyl-l-tyrosine. Prepared from *l*-tyrosine [cf. du Vigneaud & Meyer, 1932].

ON-Diacetyl-dl-tyrosine. Prepared from *l*-tyrosine [cf. du Vigneaud & Meyer, 1932].

The *O*-acetyl group of this compound may be completely split off by keeping it in excess of 0.005 *N* NaOH at 20° for 10 hr. The *N*-acetyl group is unaffected by this treatment.

**Acetyl-l-valine*. Prepared from *l*(+)-valine (sold by Hofmann-La Roche as "*d*-valin"). The product was recrystallized from water. m.p. 157–158°. $[\alpha]_D^{20} + 5.8^\circ$ (alcohol, $l=2$, $c=1.7$). (Found: C, 53.8; H, 8.29; N (Dumas), 8.5%. $C_7H_{13}O_3N$ requires C, 52.8; H, 8.17; N, 8.8%. Acid equiv. wt.: found 163; calc. 159.)

**Acetyl-dl-valine*. Prepared from synthetic *dl*-valine, the gift of Dr B. C. J. G. Knight, to whom I express my thanks. The product was recrystallized from water. m.p. 144–146°. (Found: C, 52.8; H, 8.08; N (Kjeldahl), 8.5%. $C_7H_{13}O_3N$ requires C, 52.8; H, 8.17; N, 8.8%. Acid equiv. wt.: found 159; calc. 159.)

SUMMARY

The partition of a number of acetamino-acids between immiscible solvents has been studied.

In an Appendix, the preparation of these compounds is described. Six compounds not previously described are characterized.

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CCXXXVII. EXPERIMENTS ON AMINO-ACIDS

II. AN EXPERIMENT ON THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR N-ACETYL DERIVATIVES

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(Received 5 October 1939)

THE partition coefficients of acetamino-acids between water and chloroform have been shown in Paper I of this series to be distributed over a wide range. It should therefore be possible to make use of this property for the separation of amino-acids. By constructing an extraction train in which water and chloroform flow counter-current at a controlled flow-ratio without serious entrainment it should be possible to achieve separation of a mixture of acetamino-acids by injecting it at the middle of the train. The theory of the separation is identical with that of fractional distillation, and concrete problems lend themselves to graphical treatment on the McCabe-Thiele diagram [McCabe & Thiele, 1925]. The possibilities and advantages of extractional fractionation have been stressed by Cornish *et al.* [1934], who describe an all-metal apparatus for the purpose. Dr A. J. P. Martin (unpublished work) designed and put into operation at the Dunn Nutritional Laboratory, Cambridge, an all-glass apparatus, working with the phases of a petroleum-methyl alcohol mixture. He and I have jointly been engaged on the design and construction of a train capable of handling chloroform-water phases, but this is not completed, and the present paper is concerned with the preliminary chemical problem of converting an acid hydrolysate of a protein into a mixture of those acetamino-acids which, on the basis of the figures given in Paper I of this series, might be expected to be fractionated in such a train.

Before describing the procedure adopted, it is desirable to state the reasons why acetyl derivatives rather than other acylamino-acids are used. On general grounds, it is likely that the smaller the acyl group that is introduced into the molecule, the less will it dominate the physical properties of the resulting compound, and the greater will be the differences between the partition coefficients of successive members of a homologous series of acylamino-acids. The smallest available acyl group is formyl, but formylation of amino-acids is not a convenient or gentle process, since it requires heat and dehydrating conditions. The next smallest group, acetyl, may be conveniently introduced in aqueous solution at low temperature, and involves the use of readily available reagents. For the above reasons it was not thought very hopeful to explore the use of other acyl groups for this type of separation.

Neuberger [1938, 2] showed that in the course of acetylating arginine and histidine by ketene and NaOH in aqueous solution, considerable racemization occurs. Dr Neuberger and I accordingly investigated the action of these reagents at 0° on *l*-glutamic acid, acetyl-*l*-glutamic acid, *l*-methionine, *d*-leucine, *N*-formyl-*d*-leucine, acetyl-*d*-leucine, and *d*-norleucine. We observed in each case considerable racemization. Further details of these experiments need not be given here,

since Cahill & Jackson [1938], Jackson & Cahill [1938] and Cahill [1939] have studied the phenomenon more closely.

Du Vigneaud & Meyer [1932] have shown that acetic anhydride and NaOH used in aqueous solution at 0° acetylate without appreciable racemization. As this procedure had given uniformly satisfactory results in the acetylation of individual amino-acids, it was decided to try to apply it to a known mixture of amino-acids.

It was found, by Van Slyke amino-N determinations, that a single treatment of a solution of mixed amino-acids with acetic anhydride and NaOH resulted in the acetylation of 80–95 % of the amino groups present. The problem was to obtain from an acid hydrolysate of a protein by acetylation a reasonably high yield of those acetamino-acids which, from the evidence presented in Paper I of this series, might be expected to be capable of fractionation in a suitable chloroform-water counter-current train. It is clearly desirable that this mixture should be free from salt and from non-acetylated amino-acids. It was found that this could be satisfactorily accomplished by acidifying the acetylation mixture and subjecting it to prolonged extraction by chloroform in the Neuberger [1938, 1] extractor. The mixed acetylated and non-acetylated amino-acids remaining in the aqueous layer after extraction could be separated nearly completely from the large quantities of salt present by evaporation to dryness *in vacuo*, followed by extraction with hot alcohol. They were then acetylated again, as before, and again subjected to continuous extraction with chloroform. The whole process was repeated once more.

In the experimental part of this paper the behaviour of a known mixture of amino-acids under this treatment is described in detail. The mixture was intended to simulate a gelatin hydrolysate, and lacked cystine and tryptophan. It would obviously be desirable to repeat the experiment with a mixture containing these amino-acids, as they are responsible for much secondary change when such a mixture is boiled in acid solution.

EXPERIMENTAL

All samples used in analysis were aliquots; allowance has been made for the samples removed at each stage for analysis, and all figures given in the experimental part of the paper are therefore to be referred to the original quantities used, as detailed in Table I.

Table I. *The mixture of amino-acids used in the experiment*

Compound	N (g.)	Amino-N (g.)	N as % of total N
Glycine	1.170	1.170	31.4
<i>dl</i> -Alanine	0.354	0.354	9.5
<i>L</i> -Valine	0.062	0.062	1.7
<i>L</i> -Leucine	0.189	0.189	5.1
<i>dl</i> -Serine	0.065	0.065	1.7
<i>L</i> -Phenylalanine	0.036	0.036	1.0
<i>L</i> -Methionine	0.048	0.048	1.3
<i>L</i> -Proline	0.308	0.000	8.3
<i>L</i> -Aspartic acid	0.107	0.107	2.9
<i>L</i> -Glutamic acid	0.140	0.140	3.8
<i>L</i> -Histidine (as hydrochloride)	0.075	0.025	2.0
<i>L</i> -Tyrosine	0.076	0.076	2.0
<i>L</i> -Arginine (as hydrochloride)	0.762	0.190	20.4
<i>L</i> -Lysine (as dihydrochloride)	0.330	0.330	8.9
Total	3.722	2.792	100

The mixture of amino-acids shown in Table I was dissolved in 200 ml. 10 *N* HCl, and refluxed for 5 hr. The resulting solution was evaporated to dryness *in vacuo*. The residue was taken up in water, and again evaporated. This was repeated once more. The product was then taken up in water, and treated with strong aqueous NaOH at 0° until just alkaline to thymolphthalein. The solution was then concentrated to a medium syrup *in vacuo*, and was acetylated with 400 ml. 2*N* NaOH and 40 ml. acetic anhydride, added in small portions in the course of 45 min. The mixture was well shaken and cooled in ice during the acetylation. The resulting mixture was alkaline to thymolphthalein, and was kept at 0° overnight, to make certain that any *O*-acetyl linkage that might have been formed should be broken. (Cf. note on *ON*-diacetyl-*dl*-tyrosine in Appendix to Paper I of this series.) A Van Slyke amino-N determination indicated that 80 % of the amino groups originally present were acetylated, and this figure was unchanged by storage at 0° overnight in alkaline solution.

The mixture was then brought to pH 5 by addition of 6*N* H₂SO₄, and was concentrated *in vacuo* to about 400 ml. Further 6*N* H₂SO₄ was added until the mixture was just acid to thymol blue. The volume was now approximately 500 ml. The mixture was transferred quantitatively to the Neuberger extractor, and extracted with chloroform (Extract 1, Table II). The aqueous solution was concentrated to dryness *in vacuo*, and the residue was thoroughly extracted with 800 ml. of boiling absolute alcohol, used in five successive portions. The salt residue (Residue 1, Table II) was dissolved in water, and its N content was determined on an aliquot. The alcoholic extract was concentrated to dryness *in vacuo*, the residue was dissolved in water, made alkaline and acetylated as before. The mixture was kept alkaline at 0° overnight as before. This now also served to break down any ethyl ester groupings that had resulted from the extraction with hot absolute alcohol under acid conditions [cf. Hollander & du Vigneaud, 1931]. The degree of acetylation achieved corresponded this time to acetylation of 98 % of the amino-N present in the original mixture, including that already removed in extract 1. The solution was acidified and extracted as before; this time the chloroform was changed once in the receiver of the Neuberger apparatus, so two extracts resulted. These are referred to as extracts 2A and 2B in Table II. The process of extraction occupied 3 days, as the apparatus was turned off at night, and by the end of this period an amino-N determination showed that slight hydrolysis had occurred in the aqueous layer, the degree of acetylation now corresponding to 94 % of the amino-groups of the original mixture.

The whole process was repeated once more, giving rise to Residue 2 and Extract 3. 98 % acetylation was again obtained. The final aqueous layer was freed from salt as before, giving rise to Residue 3 and "Unextracted N compounds". Direct N determinations (Kjeldahl) were carried out on all these fractions, and are given in Table II as percentages of the original total N of the mixture. 99.3 % of the original total N was thus accounted for. The number of volumes of chloroform used for each of the four extractions is also given.

Table II. *Scheme of fractionation of the mixture*

N (as % of total N)	Vol. CHCl ₃	Extract	Residue	N (as % of total N)
20.1	19	1	↔ 1	0.1
13.2	47	2A	↔ 2	0.3
3.6	21	2B	↔	
5.3	50	3	↔ 3	0.3
↓				
"Unextracted N compounds"				56.4

Investigation of the extracts

Each chloroform extract was evaporated to dryness *in vacuo*, and the residue was dissolved in water. In no case was $\text{SO}_4^{=}$ detectable in the resulting solution. In view of the fact that the aqueous layer of the extractor was in each case a nearly saturated solution of Na_2SO_4 this demonstrates the practically complete absence of entrainment in the Neuberger apparatus. This fact has been referred to in Paper I of this series. In this case the absence of $\text{SO}_4^{=}$ from the extracts made possible the use of S balance to determine the distribution of methionine in the extracts, as this was the only other S-containing substance present.

The results of Van Slyke amino-N determinations on the aqueous solutions of the extracts are shown in Table III. This indicates that prolonged boiling of acetamino-acids in chloroform in the presence of acetic acid brings about hardly any hydrolysis.

Table III. *Amino-N determinations on the extracts*

Extract	Amino-N as % of total N of each extract
1	0.06
2A	<0.01
2B	<0.01
3	0.32

An aliquot of the aqueous solution of each extract was hydrolysed at 100° in 2N HCl until the Van Slyke amino-N (as determined after neutralization of free HCl with excess of sodium acetate) reached a constant value. In Table IV the total amino-N of each extract is shown, compared with the total N of each extract. "Non-amino-N" of each extract is found by subtracting the former of these values from the latter. It will be seen that the combined "non-amino-N" totals 354 mg., in good agreement with the proline-N of the original mixture—308 mg.

The only other "non-amino-N" present in the original amino-acids is in the form of arginine and histidine. The former of these was practically absent from the extracts (see below).

Table IV. *Amino-N of extracts after acid hydrolysis*

Extract	Amino-N mg.	Total N mg.	"Non-amino-N" mg.
1	422	716	294
2A	442	469	27
2B	118	127	9
3	166	190	24
Total	1148	1502	354

S was determined on aliquots of the extracts by the method of Bailey [1937]. The results are shown in Table V. They indicate 80 % recovery of methionine in the combined extracts.

Table V. *Total S of extracts*

Extract	S as N (mg.)
1	25.6
2A	13
2B	0
3	0
Total	38.6

(Original methionine-N of mixture = 48 mg.)

The cumulative "total N", "methionine-N" and "proline-N" values of the extracts are plotted against total volume of chloroform used for extraction in Fig. 1. The line AA' represents the total N of leucine, phenylalanine, proline, methionine, valine and alanine present in the original mixture. It will be seen that the "extracted N" reaches a value considerably above this. This is due to the extraction of considerable quantities of acetylglycine, which crystallized out in the receiver of the extractor during extractions 2B and 3. The amount extracted is much larger than would be expected from the partition coefficient

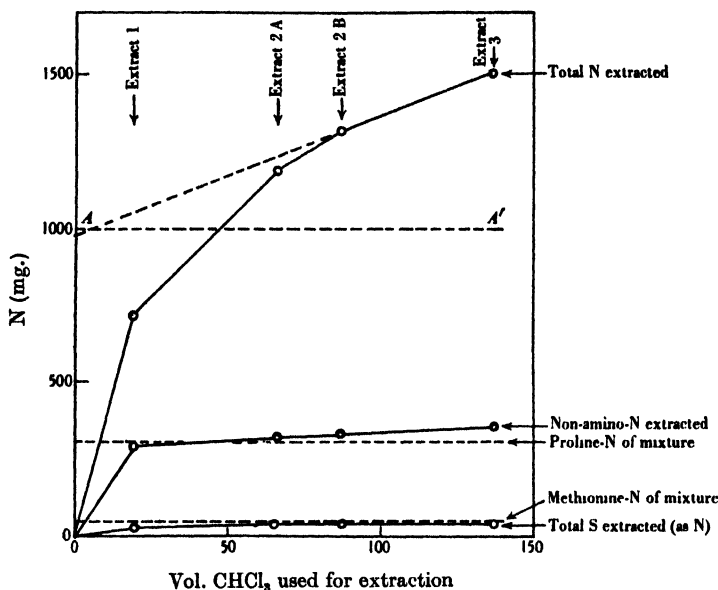


Fig. 1. Total N, non-amino-N, and S (as N) extracted from aqueous phase by chloroform after acetylation of amino-acid mixture.

determined for this substance in Paper I of this series. This is undoubtedly due to the high concentration of Na_2SO_4 in the aqueous layer of the extractor, which has a strong "salting-out effect" and is thus very favourable to the extraction, particularly as none of the salt passes into the extract by entrainment. Tyrosine was detected in all the extracts (Millon test, and yellow colour with nitrous acid). Experiments with *N*-acetyl-*L*-tyrosine showed that the "salting-out effect" with this compound is very marked, as has already been noted in Paper I of this series. The possible presence of acetyl-*L*-glutamic acid in the extracts has not been investigated.

Arginine and serine in the extracts

The acid hydrolysate of Extract 1 was tested colorimetrically for arginine by the Sakaguchi method. The test was completely negative. Addition to the test solution of arginine corresponding to the presence in Extract 1 of 0.02% of the arginine originally present in the mixture resulted in a positive test. From this it is argued that Extract 1 (which contained 20.1% of the original total N of the mixture) contained less than 0.02% of the original arginine.

Dr D. D. Woods, while working in this laboratory, observed that *Clostridium Welchii* decomposes serine under anaerobic conditions, liberating H_2 . This does not occur with any of the other compounds present in the mixture of Table I.

Dr Woods carried out bacterial tests on the acid hydrolysate of Extract 1, after it had been neutralized to pH 7 with NaOH. He showed manometrically that no gas evolution occurred, and that addition to the test solution of an amount of *dl*-serine corresponding to the presence in Extract 1 of 1 % of the serine originally present in the mixture resulted in a significant evolution of gas. From this it is argued that < 1 % of the serine originally present found its way into Extract 1. This is in agreement with the experiments on *N*-acetyl-*dl*-serine referred to in Paper I of this series.

SUMMARY

1. The possibility of separating acetamino-acids by making use of their different partition coefficients between chloroform and water is discussed.

2. In order to make this technique available for protein analysis it would first be necessary to acetylate the amino-acids of the protein hydrolysate, and to free the resulting acetyl compounds from free amino-acids and from salt.

3. A technique for carrying out this operation is outlined, and an experiment with a known mixture of amino-acids is described. Evidence is presented that those amino-acids that would be susceptible to separation by extractational fractionation are obtained in high yield in the desired state.

I wish to express my thanks to Dr K. Bailey for instructing me in the technique of S determination, to Mr N. W. Pirie for a gift of *l*-methionine, and to Dr D. D. Woods for carrying out the manometric tests for serine.

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CCXXXVIII. EXPERIMENTS ON AMINO-ACIDS

III. A METHOD FOR THE ISOLATION OF HYDROXY-AMINO-ACIDS FROM PROTEIN HYDROLYSATES

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FOUR hydroxyamino-acids may now, by the criteria of Vickery & Schmidt [1931], be regarded as authentic protein constituents. These are hydroxyproline, serine, threonine and tyrosine. The status of β -hydroxyglutamic acid must still be regarded as dubious. A number of other compounds belonging to this group have been reported as occurring in protein hydrolysates, but have not been authenticated [Dunn, 1938].

No general method for the isolation of this group of compounds has yet been described, and it is probably for this reason that so much confusion exists. Isolation in this group has hitherto depended on the chance insolubility of the amino-acid or a derivative, or on a laborious process of fractional crystallization. The isolation of threonine by Sharp [1939] from a Fischer ester distillate suggests the possibility of extending the use of this technique. A possible approach to the problem not involving isolation has been indicated by Nicolet & Shinn [1939], who have studied the reaction of serine and threonine with periodic acid. It would clearly be desirable if some method of isolation could be found which made use of the chemical function of the hydroxyl group of these compounds.

In Papers I and II of this series reference has already been made to the fact that *N*-acetyl-hydroxyamino-acids are practically unextractable from aqueous solution into chloroform. The possibility of reversing this state of affairs by benzoylating the hydroxyl group of these compounds was therefore considered. The use of benzoyl chloride in pyridine, as for the benzoylation of hydroxyl groups in carbohydrates, gave rise to undesirable coloured products. The use of pyridine was also to be avoided if it was wished to study the distribution of N in the fractions from a protein hydrolysate.

Sørensen & Andersen [1908] studied the benzoylation of some hydroxyamino-acids by benzoyl chloride and NaOH in aqueous solution. They found that:

(1) Benzoylation in the presence of excess alkali results in the formation of the *N*-benzoyl derivative.

(2) Benzoylation of the free amino-acid or its *N*-benzoyl derivative at 0° in the absence of excess alkali results largely in the formation of the *ON*-dibenzoyl derivative.

(3) Saponification in dilute aqueous alkali at room temperature converts the *ON*-dibenzoyl derivative into the *N*-benzoyl derivative.

In view of this it was decided to investigate the possibility of benzoylating the hydroxyl groups of *N*-acetyl-hydroxyamino-acids by the action of benzoyl chloride and NaOH at neutral reaction. Serine and hydroxyproline were investigated. The amino-acids were acetylated by the method of du Vigneaud & Meyer [1932] and the resulting alkaline mixture was kept for a time to ensure saponification of any *O*-acetyl groups that might have resulted. No attempt was

made to work up the resulting *N*-acetyl compound. The mixture was neutralized, and benzoylated directly, under the conditions described by Sørensen & Andersen for *O*-benzoylation. In each case a good yield of the *N*-acetyl-*O*-benzoyl derivative was obtained. These compounds are highly crystalline, and are readily extractable from aqueous solution into chloroform. Dilute aqueous alkali at room temperature rapidly converts them to the *N*-acetyl derivatives, liberating benzoic acid. Bergmann & Miekeley [1924] have shown that when *O*-benzoyl-*dl*-serine is treated with dilute aqueous alkali at room temperature acyl migration occurs, resulting in the formation of *N*-benzoyl-*dl*-serine. The present experiments show that when the amino-group is already acetylated this migration does not occur, and saponification proceeds in a normal manner. This fact is essential for the success of the method of isolation outlined below.

The behaviour on acid hydrolysis of *N*-acetyl-*O*-benzoyl-*l*-hydroxyproline was also investigated. On refluxing this compound with acid until the mixture showed constant optical rotation, there resulted a mixture of *l*-hydroxyproline and a compound which was thought to be *O*-benzoyl-*l*-hydroxyproline. Confirmation of the suggested structure of this compound was obtained by analogy: acid hydrolysis of *N*-acetyl-*O*-benzoyl-*dl*-serine yielded the known compound *O*-benzoyl-*dl*-serine, which had been described by Bergmann & Miekeley [1924].

This preferential removal of *N*-acyl groups in acid hydrolysis is probably to be explained as follows: removal by acid hydrolysis of the *N*-acetyl group sets free a positively charged NH_3^+ ($:\text{NH}_2^+$) group, which lowers the H^+ concentration in its immediate neighbourhood and thereby inhibits hydrolytic removal of the *O*-benzoyl group. A closely analogous effect was observed by Moggridge & Neuberger [1938] (cf. also Neuberger & Pitt-Rivers [1939]) in the case of the hydrolysis by acid of *N*-acetyl-methylglucosaminide.

The acid hydrolysis of *ON*-dibenzoylserine and *ON*-dibenzoyltyrosine was studied by Goldschmidt & Fünér [1930], who observed incomplete liberation of benzoic acid in each case. But as the hydrolytic agent employed was 70% H_2SO_4 at 100° , it seems likely that the phenomenon is not the same as that described here; Goldschmidt & Fünér suggested the possibility of intramolecular anhydride formation.

The procedure described here seems likely to afford a general method (at least in cases where the hydroxyl and amino groups are close to one another) for preparing *O*-benzoyl-hydroxyamino-acids, a class of compound of which the only member hitherto described is *O*-benzoyl-*dl*-serine, which was prepared by Bergmann & Miekeley [1924], by acid hydrolysis of 2-phenyl-oxazoline-4-carboxylic acid.

In attempting to make use of these results for the isolation of hydroxyamino-acids from a protein hydrolysate, the following procedure was used.

After removal of the bases by precipitation with phosphotungstic acid, the hydrolysate is acetylated by the method of du Vigneaud & Meyer [1932], and the resulting mixture, after being kept alkaline overnight, is benzoylated at neutral reaction under the conditions described by Sørensen & Andersen [1908]. The resulting mixture might be expected to contain acetamino-acids (A), *N*-acetyl-*O*-benzoyl-hydroxyamino-acids (B), and benzamino-acids resulting from benzoylation of amino-groups that have escaped acetylation (C). Benzoic acid is also present. The mixture is acidified, and extracted continuously in the Neuberger [1938] apparatus, with sufficient chloroform to ensure fairly complete extraction of *N*-acetyl-*O*-benzoylserine. The chloroform extract would now be expected to contain some of the components of group A, most of group B, most of group C and benzoic acid. The chloroform is removed by evaporation, and

the residue is dissolved in dilute alkali at room temperature. This saponifies the *O*-benzoyl group of compounds in group B, leaving the other compounds unaffected. Subsequent acidification of the mixture and prolonged extraction by chloroform removes the components of group A, group C and the benzoic acid, leaving a solution consisting largely of *N*-acetyl-hydroxyamino-acids. The free amino-acids are prepared from this by acid hydrolysis.

The results of applying this procedure (of which full details are given in the experimental section of the paper) to hydrolysates of fibrin (pig), wool (Merino 64s, dry-combed top) and gelatin (Coignet "Gold Label"), were as follows: the final fraction contained in the case of fibrin 6% of the total N of the protein, of which 89% was in the form of amino-N; in the case of wool, 5.3% (96% amino-N); and in the case of gelatin 10% (33% amino-N).

The free amino-acids of this fraction crystallized fairly readily on addition of alcohol to an aqueous solution. (Tyrosine was first obtained from fibrin and wool by direct crystallization from water.) The crystalline products were obviously mixtures, of which the N content usually lay between the theoretical values for serine and threonine. At the suggestion of Mr J. H. Humphrey, mechanical separation by "float-and-sink" analysis was attempted. A mixture of benzene and carbon tetrachloride was found which gave good flotation separation of a finely ground powder of the crystals. Recrystallization of the "top" fraction resulted, however, in a product which again gave two fractions when subjected to analysis in the same density liquid. This suggested that mixed crystals might be present, and further attempts at isolation by crystallization were abandoned.

In paper IV of this series a possible approach to the problem of separating mixtures of hydroxyamino-acids is discussed.

In the case of gelatin, the low amino-N content of the hydroxyamino-acid fraction suggests the presence of hydroxyproline, which gelatin is known to contain in large quantities. *L*-Hydroxyproline was readily isolated from this fraction by precipitation with phosphotungstic acid [cf. Kapfhammer & Eck, 1927].

It was later observed that after acetylating and benzoylating a gelatin hydrolysate from which arginine had been removed by precipitation with phosphotungstic or flavianic acids, *N*-acetyl-*O*-benzoyl-*L*-hydroxyproline crystallized out directly on acidification. This substance can readily be purified by recrystallization, and can be converted into *L*-hydroxyproline by alkaline followed by acid hydrolysis. This affords a method for the preparation of hydroxyproline which may prove of practical value as the existing methods leave much to be desired. Although the yield of hydroxyproline is only about 5% of the weight of gelatin used, no expensive reagents are required for the preparation. Full details are given in the experimental section of the paper.

EXPERIMENTAL

N-Acetyl-*O*-benzoyl-dl-serine (I)

1 g. dl-serine (Hofmann-La Roche) was dissolved in 5 ml. 2*N* NaOH and acetylated with 26 ml. 2*N* NaOH and 2.6 ml. of acetic anhydride added in portions at 0°. A Van Slyke amino-N determination on the resulting mixture indicated 94% *N*-acetylation. The mixture was kept at 0° overnight, and was then neutralized to phenol red with acetic acid. It was then placed on ice in the shaker, and treated with 37 ml. 2*N* NaOH and 4.6 ml. of benzoyl chloride, added in 10 equal portions at 30 min. intervals. The solution was made neutral to

phenol red with acetic acid before each addition of the reagents. The solution was then acidified to thymol blue with 6*N* H₂SO₄. The resulting precipitate was found to contain 80 % of the N present. It was dried, and extracted with ligroin at 100°. The insoluble residue was recrystallized from alcohol. Yield 1.6 g. *N*-acetyl-*O*-benzoyl-*dl*-serine has m.p. 192–194° (Found: C, 56.7; H, 5.25; N (Kjeldahl), 5.62, 5.66 %. C₁₂H₁₃O₅N requires C, 57.3; H, 5.18; N, 5.57 %.)

The compound has a solubility in water at 20° of 0.15 mg. per ml. Its solubility in chloroform at the same temperature is slightly greater. Its partition coefficient between chloroform and water is therefore approximately 1.

An attempt to use NaHCO₃ in excess instead of NaOH in the benzoylation led to a complete failure to prepare the compound. The yield obtained is significantly less if the benzoylation mixture is not thoroughly cooled in ice.

N-Acetyl-*dl*-serine (II)

Compound (I) was completely debenzoylated by saponification for 4 hr. in excess of 0.1*N* NaOH at room temperature. A Van Slyke amino-N determination on the resulting solution was completely negative. Chloroform extraction of the solution after acidification yielded benzoic acid; the chloroform extract was completely free from N and contained no material not volatile from an evaporating dish maintained in air at 100°. In order to isolate *N*-acetyl-*dl*-serine, Ba(OH)₂ was employed instead of NaOH; after saponification, H₂SO₄ exactly equivalent to the Ba(OH)₂ used was added, and the resulting BaSO₄ and benzoic acid were filtered off. The filtrate was extracted with chloroform to remove last traces of benzoic acid, and was then evaporated to dryness *in vacuo*. The product was a resin, soluble in water and alcohol, slightly soluble in ethyl acetate and acetone, and insoluble in other organic solvents. No attempt was made to characterize the compound further, as all attempts to make it crystallize were unsuccessful. The preparation from it of a crystalline derivative is described in Paper IV of this series.

The isolation of (II) through its benzoyl derivative is more satisfactory than its direct isolation from the acetylation mixture, since in the latter preparation it is difficult to ensure separation of the compound from serine that has escaped acetylation.

N-acetyl-*O*-benzoyl-*l*-hydroxyproline (III)

l-Hydroxyproline (Hofmann-La Roche) was treated with the same molecular proportions of reagents as employed in the preparation of (I) from serine. The product, isolated in the same manner, was recrystallized from water containing 20 % of alcohol, to aid solution. m.p. 185–186°. $[\alpha]_D^{20} - 42.9^\circ$ (alcohol, *l*=2, *c*=1.1). (Found: C, 61.2; H, 5.46; N (Kjeldahl), 4.84, 4.96 %. C₁₄H₁₅O₅N requires C, 60.6; H, 5.42; N, 5.05 %.)

The compound is readily extractable from water into chloroform. It may be recrystallized from alcohol by the addition of ether, in which it is sparingly soluble.

N-acetyl-*l*-hydroxyproline (IV)

This compound was prepared from (III) in exactly the same manner as (II) was prepared from (I). It crystallized from moist ethyl acetate as the *monohydrate*, m.p. 74–76°. This readily lost water in a vacuum desiccator at room temperature, to give the anhydrous compound, m.p. 133–134°. $[\alpha]_D^{20} - 116.5^\circ$ (water, *l*=2, *c*=3.2). (Found: C, 48.25; H, 6.71; N (Kjeldahl), 7.6 %. C₇H₁₁O₄N requires C, 48.5; H, 6.36; N, 8.1 %.)

On exposure to an atmosphere saturated with water vapour, a sample of the anhydrous product increased in weight by 9.65 %. The theoretical increase for formation of a monohydrate is 10.4 %.

Regeneration of l-hydroxyproline from (III)

For this purpose no attempt was made to work up the intermediate (IV). (III) was saponified for 4 hr. in excess of $N/3$ $Ba(OH)_2$ at room temperature; H_2SO_4 was added to make the mixture $2N$, the mixture was filtered, and was refluxed for 3 hr. It was then cooled, benzoic acid was removed by chloroform extraction, and SO_4^{2-} was removed exactly from the aqueous layer by $Ba(OH)_2$. The filtrate from $BaSO_4$ was concentrated to a very small volume *in vacuo* and, on addition of alcohol, *l*-hydroxyproline crystallized immediately. The overall yield from (III) was 80 %.

Acid hydrolysis of (III): O-benzoyl-l-hydroxyproline (V)

An attempt was made to shorten the procedure for regenerating *l*-hydroxyproline from (III) by using acid hydrolysis alone. 5.3 g. of (III) were refluxed for 3 hr. with 200 ml. $2N$ H_2SO_4 , by which time, as judged from a preliminary hydrolysis using a lower concentration of (III), the optical rotation of the mixture had reached a constant value. On cooling, the mixture was extracted with chloroform and was worked up for hydroxyproline as above. The resulting crystalline product had $[\alpha]_D^{20} - 58^\circ$ (water, $l=2$, $c=4$), and tasted bitter as well as sweet. Lutz & Jirgensons [1931] give the optical rotation of *l*-hydroxyproline as $[\alpha]_D^{20} - 76.3^\circ$ (water, $c=6.5$). *l*-hydroxyproline tastes sweet.

On recrystallization of the product from a minimum of hot water, precipitate A and mother-liquor B resulted. Recrystallization of A from water to constant m.p. gave 0.35 g. of *O*-benzoyl-*l*-hydroxyproline monohydrate. The compound does not lose water readily over H_2SO_4 *in vacuo* at room temperature. On heating, it loses water first, and then decomposes at 220° . $[\alpha]_D^{20} - 5.4^\circ$ (water, $l=2$, $c=1.2$). (Found: C, 57.0; H, 5.83; N (Dumas), 5.20 %. $C_{12}H_{13}O_4N$, H_2O requires C, 57.0; H, 5.93; N, 5.53 %.) On drying at 100° *in vacuo* over P_2O_5 the compound lost 6.2 % of its weight. (Calc. for monohydrate, 7.1 %.) The compound tasted bitter.

On addition of alcohol to mother-liquor B, crystallization of 0.95 g. of *l*-hydroxyproline resulted. $[\alpha]_D^{20} - 75.2^\circ$ (water, $l=2$, $c=5$).

O-benzoyl-dl-serine (VI)

0.37 g. of (I) was refluxed for 1.5 hr. with 10 ml. N H_2SO_4 and sufficient alcohol to effect solution. The mixture was cooled and extracted with chloroform. The aqueous layer was added to a strong solution of hydrated sodium acetate (2.7 g.), and the resulting mixture was evaporated slowly in a desiccator. Crystallization soon set in and, when crystals of hydrated Na_2SO_4 began also to appear, the mixture was warmed to 30° to dissolve these, and filtered. The crystals so obtained were recrystallized once from water. Yield 0.045 g. of *O*-benzoyl-*dl*-serine, m.p. (decomp.) 149° . Bergmann & Miekeley [1924] give m.p. (decomp.) $149-150^\circ$. (Found: C, 57.2; H, 5.59; N (Dumas), 6.57 %. Calc. for $C_{10}H_{11}O_4N$: C, 57.4; H, 5.26; N, 6.70 %.)

The isolation of a hydroxyamino-acid fraction from protein hydrolysates

20–25 g. of protein were hydrolysed by refluxing with 200 ml. $6N$ H_2SO_4 . The mixture was cooled, diluted and treated with a strong aqueous solution of phosphotungstic acid (75 g.). The final volume was 11. After keeping overnight

in the ice chest the precipitate was filtered off, and the filtrate was treated with $\text{Ba}(\text{OH})_2$ at 100° until permanently alkaline to thymol blue. The resulting precipitate was thoroughly washed, and the combined filtrate and washings were concentrated *in vacuo* to a thin syrup, and acetylated as above with 200 ml. 2N NaOH and 20 ml. of acetic anhydride. (This usually produced about 95 % acetylation of the amino-groups present.) The mixture was kept at 0° overnight, and was then neutralized to phenol red with acetic acid. Benzoylation was then carried out exactly as described for the preparation of (I), using in all 185 ml. 2N NaOH and 23 ml. of benzoyl chloride. The resulting mixture was acidified to thymol blue with 10N HCl, and filtered. The precipitate was dried in a vacuum desiccator; the filtrate was subjected to extraction by about 5 vol. of chloroform in the Neuberg extractor. When the precipitate was dry, it was exhaustively extracted with ligroin at 100° , and the insoluble residue was suspended in water in a separating funnel, and extracted with several volumes of chloroform. This extract was combined with the first chloroform extract, and evaporated to dryness *in vacuo*. The product was dissolved in 200 ml. of sat. aqueous $\text{Ba}(\text{OH})_2$ at room temperature and kept overnight. The resulting solution was practically free from amino-N. It was acidified to thymol blue with H_2SO_4 and filtered. The filtrate was extracted with about 50 vol. of chloroform in the Neuberg extractor. The aqueous layer was then concentrated *in vacuo* to about 100 ml. and treated with sufficient H_2SO_4 to render it 2N. It was then refluxed until the Van Slyke amino-N figure reached a constant value (3 hr.). SO_4^{--} was then removed exactly by $\text{Ba}(\text{OH})_2$, and the filtrate from BaSO_4 was concentrated until crystallization (first of tyrosine) began. The amounts of N and amino-N obtained in this fraction from various proteins and the attempted isolation from it of pure compounds have been described above.

Preparation of N-acetyl-O-benzoyl-l-hydroxyproline from gelatin directly

30 g. of air-dry Coignet "Gold Label" gelatin were refluxed for 6 hr. with 300 ml. 6N HCl. Excess HCl was removed by repeated evaporation with water *in vacuo*, and the product was then treated with 11 g. of flavianic acid at a volume of 150 ml. After seeding with arginine flavianate the solution was well stirred, and was kept at 0° overnight. The resulting precipitate was filtered off, and the filtrate was freed from flavianic acid by boiling with 10 g. of charcoal. The filtrate from this was made alkaline to thymol blue with NaOH, and was concentrated to a small volume *in vacuo*. Successive acetylation and benzoylation were carried out as described in the previous section. For the acetylation 400 ml. 2N NaOH and 40 ml. of acetic anhydride were used, and for the benzoylation 185 ml. 2N NaOH and 23 ml. of benzoyl chloride. The mixture was then treated with 50 ml. 10N HCl, and the resulting precipitate of benzoic acid was filtered off. On further gradual acidification of the filtrate with 10N HCl no precipitation occurred with the first additions of the acid. Later an oil appeared, which crystallized fairly rapidly. Acidification of the mixture was continued until it was acid to thymol blue. In some preparations it was advisable to seed at this stage with (III). Crystallization was allowed to continue at 0° overnight. The mixture was then filtered, and the precipitate was dried and extracted with hot ligroin. The product was then recrystallized from water containing 20 % of alcohol. Yield 2.8–3.5 g. m.p. 185° . No depression of m.p. was obtained on admixture with (III). $[\alpha]_D^{20} = -42.7^\circ$ (alcohol, $l=2, c=1.1$). (Found: C, 60.4; H, 5.22; N (Kjeldahl), 5.0, 5.3 %. Calc. for $\text{C}_{14}\text{H}_{15}\text{O}_5\text{N}$: C, 60.6; H, 5.42; N, 5.05 %.)

The preparation of *l*-hydroxyproline from (III) by alkaline followed by acid hydrolysis is described above.

If arginine is not removed from the gelatin hydrolysate before acetylation, a gummy oil is obtained as final product, in which crystals of (III) can be detected, but from which (III) can be isolated only with difficulty and in low yield.

Phosphotungstic acid can alternatively be used to remove arginine, but flavianic acid seems to lead to a better product, and is also cheaper. In some cases the final product is slightly gummy. Recrystallization from alcohol-ether is then indicated.

SUMMARY

1. The preparation and properties of the *N*-acetyl-*O*-benzoyl derivatives of *dl*-serine and *l*-hydroxyproline are described. These compounds are converted on treatment with dilute alkali into the corresponding *N*-acetyl derivatives. Acid hydrolysis yields the *O*-benzoyl derivatives.
2. On the basis of these properties, a general method is outlined for the isolation of a hydroxyamino-acid fraction from protein hydrolysates.
3. The preliminary results of such a fractionation of fibrin, wool, and gelatin hydrolysates are described.
4. A new and improved method for the preparation of *l*-hydroxyproline from gelatin has been evolved.

I am indebted to Mr B. H. Wilsdon, Director of Research, Wool Industries' Research Association, Leeds, for a supply of wool.

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CCXXXIX. EXPERIMENTS ON AMINO-ACIDS

IV. THE METHYL ETHERS OF SOME *N*-ACETYL-HYDROXYAMINO-ACIDS

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IN Paper III of this series the isolation of a "hydroxyamino-acid fraction" from protein hydrolysates is described, and it is shown that the components of this fraction are not readily separable from one another by direct crystallization. In view of the possibility of extractional fractionation of amino-acids in the form of their *N*-acyl derivatives outlined in Papers I and II of this series, it seemed desirable to search for a series of derivatives of the hydroxyamino-acids which would show a similar "spread" in their partition coefficients between a suitable pair of immiscible solvents.

The possibility of using the *N*-benzoyl derivatives was first investigated, although there are theoretical objections to introducing so heavy and fat-soluble a group. These objections were confirmed by experiment.

The ratio of the solubilities of *N*-benzoyl-*dl*-serine [Sørensen & Andersen, 1908] in water and ethyl acetate was determined. This may be taken as a measure of the partition, and can be compared with the same figures obtained for *N*-benzoyl-*dl*-threonine and *N*-benzoyl-*dl*-allothreonine by West & Carter [1937]. This is done in Table I. It seems unlikely from this that the *N*-benzoyl derivatives would be suitable for the extractional fractionation of hydroxyamino-acids.

Table I. *Solubilities of N-benzoyl-hydroxyamino-acids in ethyl acetate and water*

Compound	Temp. ° C.	Solubility in	Solubility in	B/A
		EtOAc in mg./ml. A	H ₂ O in mg./ml. B	
<i>N</i> -Benzoyl- <i>dl</i> -serine	19	20.9	25.5	1.2
<i>N</i> -Benzoyl- <i>dl</i> -threonine	25	10.2	20.6	2.0
<i>N</i> -Benzoyl- <i>dl</i> -allothreonine	25	2.4	8.5	3.5

The partition of the *N*-acetyl derivatives between ethyl acetate and water phases was next investigated. In this case the determination was carried out as in Paper I of this series, and the symbols *P* and *c* have the same meaning here as there. The "*N*-acetylthreonine" was prepared by acetylation in solution without working up. A mixture of *dl*-threonine and *dl*-allothreonine (the gift of Prof. A. C. Chibnall, to whom I express my thanks) was used as starting material. The aqueous phase for this particular determination was 0.3*N* NaCl and 0.1*N* acetic acid. The results are shown in Table II.

From this, the *N*-acetyl series seemed as unsuitable for the purpose as the *N*-benzoyl series. It seemed likely that the properties of the molecule were dominated by the presence of a free hydroxyl group, and it was therefore decided to investigate the possibilities of masking this group. Methylation, as introducing only a small group into the molecule, seemed the most hopeful line of approach. It seemed likely that the original amino-acid could be regenerated from its

Table II. *Partition of N-acetyl-hydroxyamino-acids between ethyl acetate and water*

Compound	Temp. ° C.	P	c
<i>N</i> -Acetyl- <i>dl</i> -serine	20	75	6
" <i>N</i> -Acetylthreonine"	20	45	4
<i>N</i> -Acetyl- <i>L</i> -hydroxyproline	20	50	5

N-acetyl-*O*-methyl derivative by refluxing with HBr, as in the preparation by West & Carter [1937] of very good yields of threonine isomerides in one step from their *N*-formyl-*O*-methyl derivatives.

N-Acetyl-*O*-methyl-*L*-tyrosine has been prepared from *N*-acetyl-*L*-tyrosine by methylation with aqueous NaOH and dimethyl sulphate [Karrer *et al.* 1922; Behr & Clarke, 1932]. If this method were applied to the methylation of a mixture of *N*-acetyl-hydroxyamino-acids, obvious difficulties would arise in freeing the product from excess reagents and salt. A possible alternative seemed to be the use of Purdie's reagents (silver oxide and methyl iodide). This would be expected to yield the methyl esters of the *N*-acetyl-*O*-methyl compounds, and, in view of the work of Cherbuliez *et al.* [1929; 1930] on the distillation of the ethyl esters of acetamino-acids, these should be distillable from any non-volatile reaction products. The possibility of *N*-methylation by the reagents had to be borne in mind, but since I had in the past observed that *N*-acetyl-*O*-trimethyl- β -methylglucosaminide [Cutler *et al.* 1937] results from the direct action of Purdie's reagents on *N*-acetyl-glucosamine, it seemed worth while to ascertain their effect on *N*-acetyl-hydroxyamino-acids.

It was found that, on treating *N*-acetyl-*L*-tyrosine, *N*-acetyl-*L*-hydroxyproline and *N*-acetyl-*dl*-serine with Purdie's reagents, in each case a crystalline, distillable methyl ester of the *N*-acetyl-*O*-methyl-hydroxyamino-acid resulted in fair yield. In each case this could be converted quantitatively by saponification into the free acid.

N-Acetyl-*O*-methyl-*dl*-serine was also prepared from *O*-methyl-*dl*-serine. *N*-Acetyl-*O*-methyl-*dl*-allothreonine was prepared from *O*-methyl-*dl*-allothreonine. Both these amino-acids were the gift of Dr Herbert E. Carter, to whom I express my thanks.

The partition of the *N*-acetyl-*O*-methyl-hydroxyamino-acids between chloroform and water was determined (see Paper I of this series). The results are shown in Table III.

Table III. *The partition of N-acetyl-O-methyl-hydroxyamino-acids between chloroform and water*

Compound	Temp. ° C.	P	c
<i>N</i> -Acetyl- <i>O</i> -methyl- <i>dl</i> -serine	20	450	9
<i>N</i> -Acetyl- <i>O</i> -methyl- <i>dl</i> -allothreonine	20	160	7
<i>N</i> -Acetyl- <i>O</i> -methyl- <i>L</i> -hydroxyproline	20	23	6
<i>N</i> -Acetyl- <i>O</i> -methyl- <i>L</i> -tyrosine	20	2.1	2.4

It will be seen that this series of derivatives is capable of providing the basis for extractional fractionation of the hydroxyamino-acids.

No experiments have yet been carried out on mixtures; in order to prepare the "hydroxyamino-acid fraction" (see Paper III of this series) of a protein hydrolysate for such a fractionation, a possible line of approach would be to remove SO_4^{2-} exactly with Ba from the aqueous layer after the second chloroform extraction had been carried out. The resulting solution would be evaporated to

dryness, and methylated with Purdie's reagents. The product would be distilled. The distillate would be saponified by $\text{Ba}(\text{OH})_2$, and after exact removal of Ba by H_2SO_4 , a solution of *N*-acetyl-*O*-methyl-hydroxyamino-acids suitable for extractal fractionation should result.

EXPERIMENTAL

N-Acetyl-*O*-methyl-*l*-tyrosine methyl ester (I)

1 g. of *N*-acetyl-*l*-tyrosine was dissolved in 6 ml. of dry acetone and treated for 2 hr. at 37° with 6 ml. of methyl iodide and 6 g. Ag_2O . The mixture was filtered, the insoluble material was well washed with acetone and the combined filtrate and washings were evaporated to dryness *in vacuo*. The residue was again treated with 6 ml. of methyl iodide and 6 g. Ag_2O at 37° . The mixture was filtered and evaporated to dryness as before. On distillation, 0.9 g. of material distilled at $180\text{--}200^\circ/0.05$ mm. The *product* crystallized in the receiver, and recrystallization from ether yielded 0.6 g. m.p. $106\text{--}107^\circ$. $[\alpha]_D^{20} + 26.3^\circ$ (alcohol, $l=2$, $c=4.2$). (Found: C, 61.9; H, 7.10; N, 5.64; OMe, 24.3%. $\text{C}_{13}\text{H}_{17}\text{O}_4\text{N}$ requires C, 62.1; H, 6.77; N, 5.58; OMe, 24.7%.)

N-Acetyl-*O*-methyl-*l*-tyrosine (II)

(I) was dissolved in excess of *N* NaOH and kept for 4 hr. at room temperature. On acidifying with HCl, crystallization occurred, and the product on recrystallization from water had m.p. 151° (not depressed on admixture with a sample of *N*-acetyl-*O*-methyl-*l*-tyrosine provided by Prof. H. T. Clarke, to whom I express my thanks).

$[\alpha]_D^{20} + 54.3^\circ$, $+54.4^\circ$; $[\alpha]_{5491}^{20} + 65.9^\circ$ (alcohol, $l=2$, $c=1.3$). (Behr & Clarke [1932] record m.p. $150\text{--}151^\circ$; $[\alpha]_{5461}^{20} + 67.6^\circ$ (alcohol, $c=5$)). (Found: C, 60.5; H, 6.21; N, 5.84; OMe, 13.4%. Calc. for $\text{C}_{12}\text{H}_{15}\text{O}_4\text{N}$: C, 60.8; H, 6.33; N, 5.90; OMe, 13.1%. Acid equiv. wt. Found: 231. Calc.: 237.

N-Acetyl-*O*-methyl-*l*-hydroxyproline methyl ester (III)

1.4 g. of *N*-acetyl-*l*-hydroxyproline (see Paper III of this series) were methylated as in the preparation of (I), using double the quantities of reagents. It was advisable to grind the starting material to a fine powder, as it is not very soluble in acetone. 1.24 g. of distillate ($125\text{--}150^\circ/0.05$ mm.) were obtained, which crystallized immediately. Recrystallization from ether yielded a *product* with m.p. $76\text{--}77^\circ$. $[\alpha]_D^{18} - 81.0^\circ$ (alcohol, $l=2$, $c=4.5$). (Found: C, 54.1; H, 7.44; N, 6.91; OMe, 31.2%. $\text{C}_9\text{H}_{15}\text{O}_4\text{N}$ requires C, 53.8; H, 7.46; N, 6.96; OMe, 30.8%.)

N-Acetyl-*O*-methyl-*l*-hydroxyproline (IV)

1.0 g. of (III) was dissolved in 50 ml. $N/3$ $\text{Ba}(\text{OH})_2$ and was kept at room temperature for 3 hr. Ba was then removed exactly with H_2SO_4 , and the filtrate from BaSO_4 was evaporated to dryness *in vacuo*. The *product* was crystallized from chloroform by addition of ether, and was recrystallized from a minimum of water, in which it is very soluble. m.p. $152\text{--}153^\circ$; $[\alpha]_D^{18} - 104.3^\circ$ (water, $l=2$, $c=3$). (Found: C, 51.5; H, 7.03; N, 7.47; OMe, 16.9%. $\text{C}_8\text{H}_{13}\text{O}_4\text{N}$ requires C, 51.3; H, 6.95; N, 7.48; OMe, 16.6%.) Acid equiv. wt. Found: 184. Calc.: 187.

N-Acetyl-*O*-methyl-*dl*-serine methyl ester (V)

1.18 g. of *N*-acetyl-*dl*-serine (see Paper III of this series) were methylated with the same amounts of reagents as in the preparation of (III). As the starting material is a glass of low solubility in acetone, it was obtained in a finely divided

state by evaporating a strong aqueous solution with kieselguhr in a mortar in a vacuum desiccator. When dry, the product was thoroughly ground. The resulting powder was transferred to the reaction vessel and thoroughly dried in the desiccator. After methylation, distillation (100–140°/0.05 mm. Hg) yielded 0.9 g. of a product which crystallized in the receiver on keeping overnight. Recrystallization from ether (in which the compound was rather soluble) yielded 0.4 g. m.p. 70–71°. (Found: C, 48.3; H, 7.25; N, 7.95; OMe, 35.0%. $C_7H_{13}O_4N$ requires C, 48.0; H, 7.43; N, 8.00; OMe, 35.4%.)

N-Acetyl-O-methyl-dl-serine (VI)

This compound could be made by saponification of (V) but it was found that the crude distillate containing (V) on saponification yielded a contaminant which rendered the crystallization of (VI) rather difficult. This contaminant could be removed by prolonged extraction of an aqueous solution of the saponification product with chloroform. I am grateful to Mrs R. V. Pitt Rivers for carrying out this extraction. The compound could be more conveniently prepared by direct acetylation of O-methyl-dl-serine, using the procedure described in the Appendix to Paper I of this series. The compound crystallized very slowly from ethyl acetate. m.p. 108–109°. (Found: C, 43.8; H, 6.95; N, 8.42; OMe, 18.1%. $C_6H_{11}O_4N$ requires C, 44.7; H, 6.82; N, 8.69; OMe, 19.2%.) Acid equiv. wt. Found: 165. Calc.: 161.

N-Acetyl-O-methyl-dl-allothreonine (VII)

This compound was prepared in the same way as (VI) by direct acetylation of O-methyl-dl-allothreonine [West & Carter, 1937]. It was recrystallized from acetone. m.p. 151°. (Found: C, 47.9; H, 7.77; N, 7.69; OMe, 18.1%. $C_7H_{13}O_4N$ requires C, 48.0; H, 7.43; N, 8.00; OMe, 17.7%.) Acid equiv. wt. Found: 170. Calc.: 175.

SUMMARY

The preparation and properties of some N-acetyl-O-methyl-hydroxyamino-acids are described.

The partition coefficients of these compounds between chloroform and water have been measured.

It is suggested that, by means of these derivatives, extractational fractionation of the hydroxyamino-acids from protein hydrolysates might be accomplished.

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CCXL. NEW ZEALAND FISH OILS

III. THE COMPOSITION OF THE DEPOT FATS OF THE LING (*GENYPTERUS BLACODES*)

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THE available data on marine animal fats indicate that the composition of the fatty acids of a given species varies according to the depot from which the samples were taken [cf. Hilditch & Lovern, 1928; Lovern, 1934; 1937; Shorland & Hilditch, 1938]. Lovern [1934] suggested that one of the mechanisms controlling selective deposition of fat depends on molecular size and is probably concerned with molecular filtration, whereby acids of lower molecular weight are permitted to enter all depots with equal facility while those of higher molecular weight are not so readily admitted to the less permeable depots. Whatever the mechanism of selective deposition, the data so far obtained generally show a correlation between the size and fat content of the depot of a given species and the composition of the fatty acids. In this connexion the ling is especially interesting as the fat is concentrated almost entirely in the liver [Shorland, 1937], the other depots being presumably relatively impermeable and therefore likely to show to a marked degree the effects of selective deposition of the fatty acids.

It has been shown [cf. especially Harper & Hilditch, 1937; Hilditch & Terleski, 1937; Lovern, 1938] that the composition of oil from a given species may vary considerably according to locality and season. The analysis of one sample only may not therefore give a satisfactory picture of the general composition of a particular depot fat within a given species. In this investigation 7 samples of ling liver oil, collected at different periods from Cook Strait, and the viscera and roe lipins previously described [Shorland, 1938] were analysed by the ester fractionation method. The general characteristics of these oils and also of a specimen of oil extracted from a whiptail (*Macruronus novae zelandiae*), the chief source of food of the ling, are given in Table I.

The liver oils (1-3) were analysed according to the procedure of Guha *et al.* [1930], but for the remaining samples advantage was taken of the improved technique of Harper *et al.* [1937] whose methods of calculation were followed throughout. 100-300 g. oil were taken for ester fractionation wherever possible. In the case of the viscera and roe lipins, however, 5-8 g. only of the sample were available, and the esters were fractionated in a Vigreux column (26 × 1 cm.) with a bulb capacity of 20 ml. The column was lagged with asbestos string and heated in a glycerol bath contained in a wide test tube (20 × 5 cm.). As the distillation proceeded the column was suitably lowered into the bath to facilitate fractionation of the higher boiling point esters. The iodine values and saponification equivalents of the fractions obtained with this column were determined by standard methods using a 10 ml. microburette and reduced amounts of reagent. In Table II the efficiency of this column is compared with that of a 250 ml. Willstätter flask packed as described by Hilditch & Houlbrooke [1928].

Table I. *General characteristics of ling (Genypterus blacodes) lipins*

Depot	Date of catch	Mean wt. tissue per fish g.	Lipid %	No. of specimens	Blue value	Sap. equiv.	I.V. (Wijs 1 hr.)	Un-saponifiable matter %	P %	Free fatty acid (as oleic acid) %
Liver (1)	31. i. 35	453	35-40	17	780	301.9	142.9	3.05	Trace only	—
(2)	6. vi. 35	580		94	430	303.0	136.5	3.37		—
(3)	9. vii. 34	525		36	420	304.6	142.0	3.10		0.31
(4)	28. vii. 38	—		(Bulk sample)	410	302.0	146.6	2.95		—
(5)	2. viii. 35	648		28	230	300.9	142.2	2.11		—
(6)	22. viii. 35	942		25	320	303.9	139.8	2.23		—
(7)	4. ix. 35	—		(Bulk sample)	420	306.8	142.6	2.87		—
Viscera (excluding liver)	2. vii. 35	1210	0.6	2	820	357.2	178.8	18.2	0.14	34.9
Roe glyceride	28. ix. 35	566	0.5	4	No test	387.2	169.6	18.3	3.74	—
Roe phosphatide	28. ix. 35		0.4			302.1	150.2			
Whiptail (whole fish)	2. vii. 35	1246	0.7	1	75	303.0	143.6	4.79	0.047	3.0

Table II. *Comparative distillation data for methyl esters of "liquids" of ling liver oil 5*

Willstätter bulb				Semi-micro column			
Wt. g.	B.P. (0.1 mm.)	S.E.	I.V.	Wt. g.	B.P. (0.1 mm.)	S.E.	I.V.
L 1 4.73	103/129°	275.1	77.2	L 1 1.20	75/109°	277.4	88.7
L 2 3.83	129/130°	287.0	103.5	L 2 1.13	109/117°	286.4	99.2
L 3 3.83	130/132°	294.2	117.3	L 3 1.55	117/130°	299.1	129.6
L 4 4.06	132/137°	297.6	132.3	L 4 0.79	130/134°	302.6	159.4
L 5 4.05	137/145°	306.4	183.2	L 5 1.09	134/142°	324.2	264.5
L 6 5.46	145/155°	325.8	277.0	L 6 0.37	142°/falling	331.6	319.5
L 7 4.20	—	350.5	315.8	L 7 0.80	—	351.6	308.5
30.16		(332.2)*		6.93		(332.2)*	

* Saponification equivalents of residual esters freed from unsaponifiable matter.

The "solids" were not fractionated, but for the purposes of comparison the composition was ascertained by attributing the small iodine value of the "solids" (3.6) to oleic acid and expressing the results in terms of saturated and component unsaturated acids.

Table III. *Effect of method of fractionation on the calculated composition of ling liver oil 5*

Method	Fatty acids (wt. %)				
	Saturated	Unsaturated			
		C ₁₆	C ₁₈	C ₂₀	C ₂₂
Simple fractionation by semi-micro column	20.9	9.1 (2.0)*	37.3 (2.8)	23.0 (6.0)	9.7 (10.8)
Simple fractionation by Willstätter bulb	21.4	8.4 (2.0)	37.5 (2.8)	22.9 (6.2)	9.8 (10.0)
Detailed fractionation according to procedure of Harper <i>et al.</i> [1937]	21.4	8.9 (2.0)	35.4 (2.8)	25.4 (5.5)	8.9 (10.0)

* Figures in brackets indicate mean unsaturation expressed in terms of hydrogen.

Although it is not possible to obtain a precise estimate of the accuracy of simple fractionation for a complex oil, the data given in Table III suggest that a single distillation by means of the Willstätter bulb or by the semi-micro column gives approximately the same result as the more detailed fractionation.

To test the accuracy of the macro-fractionation process sample 2, after analysis by the usual procedure, was hydrogenated (i.v. 5.8) and again analysed by detailed fractionation using a Willstätter bulb. The analysis of another sample, no. 4, was checked by distillation of the "liquids" in a heated and packed column of the kind described by Longenecker [1937], after fractionation by the usual method employing a Willstätter bulb. As an illustration of the method used, the latter fractionation data are given in Table IV.

Table IV. *Comparison of fractionation data obtained by use of the heated and packed column and by the Willstätter bulb*

Heated and packed column				
	Wt. g.	B.P. (0.1 mm.)	S.E.	I.V.
L 1	1.81	110/111°	266.2	51.1
L 2	2.00	111/140°	277.7	72.2
L 3	3.08	140/146°	288.6	87.1
L 4	2.57	146/147°	295.6	93.1
L 5	4.97	147/149°	297.8	117.1
*L 6	3.69	149/156°	304.9	142.8
*L 7	4.94	156/166°	328.0	221.9
*L 8	3.04	166/176	345.2	314.6
*L 9	2.68	—	495.3	206.0

* Sap. equiv. of esters freed from unsaponifiable matter: L 6 304.9, L 7 322.5, L 8 339.6, L 9 340.9.

Willstätter bulb					
	Wt. g.	B.P. (0.1 mm.)	Wt. g.	B.P. (0.1 mm.)	I.V.
L 1	15.43	132/145°	L 11	2.84	269.5
			L 12	3.22	274.0
			L 13	2.97	282.4
			L 14	6.38	297.1
L 2	9.25	145/152°	L 21	2.15	284.5
			L 22	2.59	288.9
			L 23	2.38	297.2
			L 24	1.86	308.6
L 3	13.61	152/155°	L 31	2.82	296.8
			L 32	3.47	298.0
			L 33	4.48	303.2
			*L 34	2.58	315.9
†L 4	9.03	155/174°	L 41	2.03	307.4
			L 42	2.63	315.3
			L 43	2.05	318.8
			*L 44	2.16	334.9
†L 5	16.57	—	L 51	2.33	327.5
			L 52	2.18	332.0
			L 53	2.51	336.6
			*L 54	6.46	389.5

* Sap. equiv. of esters freed from unsaponifiable matter: L 34, 311.0; L 44, 323.8; L 54, 344.6.

† Unsaponifiable matter extracted prior to refractionation.

The phosphorus content of the lipins (cf. Table I) shows the absence of appreciable amount of phosphatide except in the case of the roe lipins. These were submitted to an acetone separation and the resultant glyceride and phosphatide fractions were analysed separately.

Table V. *Composition of ling (Genypterus blacodes) fats*

		(a) Fatty acids (wt. %)							
Depot	Date collected	Saturated			Unsaturated				
		C ₁₄	C ₁₆	C ₁₈	C ₁₈	C ₁₈	C ₂₀	C ₂₂	
Liver (1)	31. i. 35	1.9	16.9	2.6	6.5 (2.0)	34.9 (2.5)	25.1 (5.0)	12.1 (7.6)	
(2)	6. vi. 35	2.2	18.0	1.8	5.5 (2.0)	38.4 (2.6)	25.8 (5.4)	8.3 (6.9)	
(3)	9. vii. 34	1.3	15.8	1.2	7.8 (2.0)	37.6 (2.4)	24.4 (4.9)	11.9 (8.8)	
(4)	28. vii. 38	0.7	15.8	2.6	6.6 (2.0)	35.5 (2.6)	23.7 (5.4)	15.1 (8.5)	
(4)	28. vii. 38 (Heated and packed column)	1.1	16.4	2.6	6.4* (2.0)	37.4 (2.2)	21.9 (5.3)	14.2 (9.0)	
(5)	2. viii. 35	2.2	16.2	2.6	9.4 (2.0)	35.3 (2.7)	25.3 (5.7)	9.0 (10.0)	
(6)	22. viii. 35	2.0	15.8	3.8	7.6 (2.0)	34.3 (2.6)	23.3 (5.4)	13.2 (9.7)	
(7)	4. ix. 35 (Heated and packed column)	1.9	16.1	2.7	7.2† (2.0)	34.5 (2.1)	24.2 (5.3)	13.4 (8.5)	
Viscera (excluding liver)	2. vii. 35	0.9	18.9	2.9	6.7 (2.0)	16.9 (2.9)	36.6 (5.6)	17.1 (9.4)	
Roe (phosphatide)	28. ix. 35	1.3	25.0	0.9	2.1 (2.0)	20.2 (2.7)	34.4 (7.1)	16.1 (10.0)	
Roe (glyceride)	28. ix. 35	—	20.4	2.0	7.0 (2.0)	30.8 (3.1)	28.7 (7.3)	11.1 (7.3)	

* Includes 0.1% myristoleic acid.

† Includes 1.1% myristoleic acid.

		(b) Total groups of acids (mol. %)					
Depot	Date collected	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	
Liver (1)	31. i. 35	2.3	26.1	37.9	23.3	10.4	
(2)	6. vi. 35	2.7	26.1	40.5	23.9	6.8	
(2)	6. vi. 35	2.5	27.4	39.8	22.2	8.1	
	(Hydrogenated)						
(3)	9. vii. 34	1.6	26.6	39.4	22.9	9.5	
(4)	28. vii. 38	0.9	25.1	38.8	22.1	13.1	
(4)	28. vii. 38	1.3	25.7	40.3	20.4	12.3	
	(Heated and packed column)						
(5)	2. viii. 35	2.8	28.2	38.0	23.3	7.7	
(6)	22. viii. 35	2.5	26.1	38.3	21.7	11.4	
(7)	4. ix. 35	3.5	25.1	37.6	22.5	11.3	
	(Heated and packed column)						
Viscera (excluding liver)	2. vii. 35	1.2	29.0	20.3	34.5	15.0	
Roe phosphatide	28. ix. 35	1.7	30.4	21.5	32.4	14.0	
Roe glyceride	28. ix. 35	—	30.5	33.2	26.8	9.5	

(c) Deviations from mean values of total groups of acids (mol. %) of the liver oils

		C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	
Mean value	...	2.2	26.3	38.9	22.5	10.1	
Liver oil	Date collected						
(1)	31. i. 35	+0.1	-0.2	-1.0	+0.8	+0.3	
(2)	6. vi. 35	+0.5	-0.2	+1.6	+1.4	-3.3	
(2)	6. vi. 35	+0.3	+1.1	+0.9	-0.3	-2.0	
	(Hydrogenated)						
(3)	9. vii. 34	-0.6	+0.3	+0.5	+0.4	-0.6	
(4)	28. vii. 38	-0.3	-1.2	-0.1	-0.4	+3.0	
(4)	28. vii. 38	-0.9	-0.6	+1.4	-2.1	+2.2	
	(Heated and packed column)						
(5)	2. viii. 35	+0.6	+1.9	-0.9	+0.8	-2.4	
(6)	22. viii. 35	+0.3	-0.2	-0.6	-0.8	+1.3	
(7)	4. ix. 35	+1.3	-1.2	-1.3	—	+1.2	
	(Heated and packed column)						

The results in Table V give general confirmation of the accuracy of the ester fractionation technique which has already been tested for fish oils by Harper *et al.* [1937]. The outstanding feature, however, of the present investigation is the apparent difference in the values obtained for the mean unsaturation of C_{18} esters by the heated and packed column on the one hand (-2.1 to -2.2 H) and by the less efficient Willstätter bulb on the other (-2.4 to -2.7 H). It has already been shown in connexion with animal liver fats [Hilditch & Shorland, 1937] that the Willstätter bulb does not permit complete separation of C_{20} unsaturated esters from C_{18} ester concentrates which give on bromination of the corresponding acids varying proportions of high melting point bromides characteristic of highly unsaturated C_{20} acids. An investigation of the C_{18} unsaturated acids (corresponding esters, s.e. 295.4, i.v. 90.2) prepared by refractionation of a C_{18} ester concentrate in the heated and packed column gave the results reported in Tables VIa and VIb.

Table VIa. Separation of C_{18} unsaturated acids of ling liver oil by lithium salts

A. Acetone-soluble			B. Acetone-insoluble alcohol-soluble			C. Insoluble both in acetone and in alcohol		
%	S.E.	I.V.	%	S.E.	I.V.	%	S.E.	I.V.
6.0	282.4	142.2	8.1	280.5	99.0	85.9	280.6	79.4

Table IVb. Insoluble bromo-additive products of each fraction

Fraction	Insoluble in ether			Soluble in ether, insoluble in petroleum		
	M.P.	% Br	% total acids	M.P.	% Br	% total acids
A	231°	67.7	0.4	110/118°	52.3	0.5
B	224°	66.1	0.1	155°	—	ca. 0.1
C	235°	—	ca. 0.1	170/175°	59.7	0.2

The saponification equivalent of the acetone-soluble fraction A and the presence of high-melting point ether-insoluble bromides shown in Table VI suggest that even the relatively efficient heated and packed column does not separate completely in one distillation a pure C_{18} ester fraction. In order to eliminate the last traces of C_{20} esters the C_{18} concentrate was twice redistilled, giving finally a series of similar fractions.

Table VII. Distillation of C_{18} esters prepared by repeated distillation from a heated and packed column

Fraction	g.	B.P./0.1 mm.	S.E.	I.V.
C_{18} 1	2.82	134/135°	295.2	88.3
C_{18} 2	12.96	135°	295.9	89.3
C_{18} 3	2.79	—	295.9	88.2

Bromination of corresponding acids of fraction C_{18} 2 gave the results shown in Table VIII.

Table VIII. Bromination of acids from "highly purified" C_{18} esters

Ether-insoluble			Soluble in ether, insoluble in petroleum		
M.P.	% Br	% total C_{18} acids	M.P.	% Br	% total C_{18} acids
217/218°	65.6	0.4	173/174°	57.1	0.5

As a result of repeated fractionation the proportions of acids giving rise to ether-insoluble and petroleum-insoluble bromides were found to be reduced respectively from 0.6 to 0.4 % and from 0.8 to 0.5 % respectively. The % Br (65.6) accords with the presence of a mixture of octadecatetraenoic (69.8 % Br) and octadecatrienoic (63.3 % Br) acids which Toyama & Tsuchiya [1929] have shown to exist in sardine oil, the ether-insoluble bromide from the octadecatetraenoic (stearidonic) acid melting at approximately 220° as compared with 217–218° observed for the ether-insoluble C_{18} bromides reported in this investigation.

DISCUSSION

If allowance is made for experimental errors in the ester fractionation method [cf. Harper *et al.* 1937] the liver oils, except perhaps in the case of the C_{22} acids, were not found to vary appreciably in the proportions of the total groups of acids (cf. Table Vc). This observation may have considerable significance in regard to the average composition of ling liver oil, since the samples differed as regards average liver weight and covered a wide range of blue values (cf. Table I). Variations in the content of hexadecenoic and palmitic acids are suggestive of dehydrogenation and hydrogenation processes which have been inferred already by Lovern [1937] in connexion with several North Sea species.

Comparison with previous results [Shorland & Hilditch, 1938] shows that "English" hake (*Merluccius gayi*) liver oil from Cook Strait is not significantly different in composition from ling liver oil, whereas groper (*Polyprius oxygeneios*) liver oil from the same locality is characterized by greatly reduced proportions of highly unsaturated C_{20} and C_{22} acids and correspondingly increased amounts of palmitic and palmitoleic acids. In both ling and "English" hake the liver predominates as a fat depot, but in groper the liver is subordinate as a fat depot to the main fat depots of the head and body. Groper head fat [Shorland & Hilditch, 1938] has been shown to conform approximately to the "average" marine type [cf. Lovern, 1937] of the North Sea and it is therefore possible that the highly abnormal composition of the liver oil may be a result of selective deposition.

Ling liver oil is shown to conform essentially to the "average" marine type as regards fatty acid composition, with slightly increased proportions of C_{18} unsaturated acids which have been a consistent feature of all New Zealand fish oils so far examined. This suggests that the conception of an "average" marine type of fat based on analyses of specimens taken mainly from the North Sea may not be applicable to other localities, and in this connexion the presence of increased proportions of C_{18} acids in Antarctic whale oil as compared with Arctic samples [Hilditch & Terleski, 1937] may be significant.

The roe glyceride somewhat resembles the liver fat as regards fatty acid composition, while the increased proportions of C_{20} and of C_{22} unsaturated acids and the diminished proportions of hexadecenoic acid of the roe phosphatide as compared with the corresponding glyceride are in accordance with previous observations made on animal liver phosphatides [cf. Klenk, 1933; 1935; Hilditch & Shorland, 1937; Shorland & Hilditch, 1938]. Although the visceral fat contains a considerably smaller proportion of palmitic acid than the roe phosphatide, it resembles the latter as regards the proportions of the total groups of acids. It has been generally found that roe lipins are more unsaturated than the corresponding depot fats [Channon & El Saby, 1932; Lovern, 1934], especially just prior to spawning. The higher iodine number of the roe lipins does not necessarily indicate an increased content of C_{20} and C_{22} highly unsaturated acids as compared with the depot fats. The greater degree of unsaturation of the fat of salmon ova

as compared with the body fat is due to the higher mean unsaturation of the C_{20} and C_{22} acids, which are present in smaller proportions than in the body fat. In the case of the ling, however, the higher iodine value of the roe lipins as compared with the liver oil may be attributed both to increased proportions of C_{20} and of C_{22} unsaturated acids and to the higher mean unsaturation of the C_{20} acids.

In view of the low proportions of fat in the viscera and roe as compared with the liver it would be expected on the basis of Lovern's hypothesis that the former depots would be relatively impermeable to the fatty acids of higher molecular weight. Contrary to previous observations on fish oils, however, these relatively impermeable depots show no appreciable increase in the proportions of C_{16} or lower fatty acids as compared with the liver fat but a marked increase in their content of C_{20} and to a lesser extent of C_{22} highly unsaturated acids.

SUMMARY

Analyses of 7 samples of ling (*Genypterus blacodes*) liver oil taken at different periods showed no significant seasonal variation in fatty acid composition. The accuracy of the ester fractionation procedure was tested by various methods, all of which returned similar values for the proportions of component fatty acids. In the case of fractionation by a heated and packed column the calculated mean unsaturation of the C_{18} acids was much less than the value obtained by use of the less efficient Willstätter bulb. The composition of the liver oils was found to be generally similar to the "average" marine type with increased proportions of C_{18} unsaturated acids.

The iodine value and bromination data of highly purified C_{18} unsaturated acids, prepared from the corresponding methyl esters which had been repeatedly fractionated in a heated and packed column, showed that at least 96.5% of the acids consisted of octadecenoic acid together with traces of octadecatetraenoic and octadecatrienoic acids. There was no indication of the presence of either linoleic or linolenic acid.

The roe glyceride was found to contain similar proportions of component fatty acids to the liver fat and contained more hexadecenoic and less C_{20} and C_{22} highly unsaturated acids than the corresponding phosphatide.

Contrary to Lovern's hypothesis of molecular filtration, the viscera and roe, which are relatively insignificant fat depots of the ling as compared with the liver, were characterized by containing fatty acids with higher proportions of C_{20} and C_{22} unsaturated acids than the liver oil.

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CCXLI. THE SPECIFICITY OF GLUTAMINE FOR GROWTH OF *STREPTOCOCCUS HAEMOLYTICUS*

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(Received 16 October 1939)

GLUTAMINE was established as an essential nutrient for *Streptococcus haemolyticus* by McIlwain *et al.* [1939] and its importance in growth of other bacteria, notably pneumococcus, was shown by Fildes & Gladstone [1939]. It is involved in many other biological materials and processes, which makes desirable the determination of the specificity of the glutamine structure in producing these effects. The present results of attempted growth of streptococcus in the presence of compounds related to glutamine show that glutamine is extremely specific in this respect.

Conditions of testing

The bacteriological technique was that described by McIlwain *et al.* using, however, a small inoculum of approximately 1000 organisms. The compounds were tested as neutral solutions sterilized by filtration and added to the glutamine-free medium in maximum concentrations of $2 \times 10^{-3} M$ and minimum concentrations of $8 \times 10^{-5} M$ except when otherwise indicated. Incubation was continued for at least 7 days. Control tests with glutamine itself consistently attained maximum growth in 1 day with $4 \times 10^{-4} M$ solutions and in 5-7 days with $6 \times 10^{-7} M$ solutions.

Preparation and growth activity of glutamine analogues

Compounds tested are listed below in order of their deviation from the glutamine structure. When activity was observed, results of growth are given; all other specimens were inactive.

A. Compounds differing from glutamine at its amide grouping

Glutamic acid is inactive under the present conditions. Its effect under different conditions has been described by McIlwain *et al.* [1939] and by Fildes & Gladstone [1939].

α -Aminobutyric acid. A commercial specimen was used.

α - γ -Diaminobutyric acid was given by Dr R. L. M. Synge [Synge, 1939].

Glutathione. A commercial specimen was inactive under the present conditions [cf. McIlwain *et al.* 1939].

B. Compounds differing from glutamine at its amine grouping

Glutaric acid monoamide. Glutaric acid was converted to the anhydride which was distilled (B.P. 151-152°/20 mm.), crystallized (M.P. 51°) and converted to the amide (M.P. 94°, from acetone-ether). (Found: N, 10.8%. Calc. for $C_5H_9O_3N$: N, 10.7%.)

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l-Leucyl-d-glutamine was given by Prof. Chibnall.

Cysteylglutamine was given by Prof. Harington [v. Harington & Mead, 1936].

N-Acetyl-l-glutamine. Glutamine (see note *a*, p. 1946) was acetylated according to Syngé [1939] in 80 % yield. The immediate product, and a specimen given by Dr R. L. M. Syngé, caused slight growth of streptococcus in 1 day at a concentration of 2×10^{-3} *M* and in 3 days at 4×10^{-4} *M*. In view of the negative results with glutamine peptides, this was suspected to be due to contamination with glutamine, of which about 0.01 % would give the observed effect. Such was found to be the case, for on repeated crystallization from alcohol, 4-1 acetone-water and 4-1 alcohol-water, a specimen was obtained (M.P. 208°; N, 14.8 %. Calc. for $C_7H_{12}O_4N_2$, 14.9 %) which was without growth-promoting activity.

C. *Compounds altered at the carboxylic acid grouping of glutamine*

d-Glutaminylglycine and *d-glutaminy-l-d-glutamic acid* were given by Prof. Chibnall [v. Melville, 1935].

Glutaminylcysteine was given by Prof. Harington [v. Harington & Mead, 1936].

D. *Compounds altered at the amide and amine groupings of glutamine*

Pyrrolidone- α -carboxylic acid. Specimens were obtained from glutamine by the method of Vickery *et al.* [1935] and from glutamic acid according to Foreman [1914], but the following, based on a method outlined by Haitinger [1882], was found best. *dl*-Glutamic acid (20 g.) was heated in a slow stream of N_2 in a metal-bath at 190–200°. Water was evolved vigorously after about 10 min.; the temperature remained at 160° during this, but after 5 min. rose and was kept at 185–190° for 2 hr. The resulting light brown liquid was allowed to cool to 100°, water (18 ml.) and HCl (2 ml. conc.) added and the mixture filtered. Pyrrolidone- α -carboxylic acid separated on cooling; more was obtained from its mother liquors and the whole recrystallized from dilute HCl (11 g., M.P. 183°. Found: N, 10.8 %. Calc. for $C_5H_7O_3N$: N, 10.8 %).

This compound is of interest as ammonium pyrrolidone- α -carboxylate is the product of breakdown of glutamine in aqueous solution [Vickery *et al.* 1935]. It was attempted to reverse this reaction by treating the inactive ammonium salt with NH_4OH and ammonium salts under various conditions; no appreciable amount of active material was found in the products.

Glutaric acid. A commercial specimen was used.

α -Oximinoglutaric acid. Diethyl- α -oximinoglutarate [McIlwain & Richardson, 1939] was very easily hydrolysed by NaOH according to Wislicenus & Grützner [1909] to the acid, which was recrystallized from water with the minimum of heating; M.P. 154–155° with gas evolution in a sealed capillary. Found: N, 8.6 %. Calc. for $C_5H_7O_5N$: N, 8.7 %.

α -Uramidoglutaric acid. The methods of Lippich [1908] and Dakin [1919] were used to yield mixtures of the acid with unchanged glutamic acid, from which it was very difficult to separate by crystallization. The solubilities of the heavy metal salts of the uramido-acid were investigated in order to discriminate between it and pyrrolidone- α -carboxylic acid and glutamic acid. The uramido-acid only was found to be precipitated by Pb salts at pH 5.5–6. The following method takes advantage of this.

dl-Glutamic acid (10 g.) urea (25 g.) and $Ba(OH)_2 \cdot 8H_2O$ (40 g.) in water (1000 ml.) were boiled in an open flask and 2 % $Ba(OH)_2$ added to restore the volume each time the pH reached 8. $BaCO_3$ was precipitated and NH_3 evolved. When the solution remained strongly alkaline after long boiling (after the

addition of about 3 l. of solution in 60 hr.), CO_2 was passed to remove excess $\text{Ba}(\text{OH})_2$, the solution filtered and evaporated in vacuum to about 300 ml. Saturated aqueous basic Pb acetate solution was added till in excess, maintaining the solution at pH 5.5–6 by the addition of glacial acetic acid. The crystalline Pb salt was collected, suspended in warm water and H_2S passed. After removal of PbS the solution was concentrated to crystallization. Uramidoglutamic acid separated and was washed with water and alcohol: 5 g., M.P. 166° with gas evolution. Found: N, 14.8%. Calc. for $\text{C}_9\text{H}_{10}\text{O}_5\text{N}_2$: N, 14.7%. Its M.P. was not depressed by admixture with glutamic acid, but varied between 180 and 190° . *l*-Uramidoglutamic acid, similarly prepared, melted at 174° .

E. *Compounds altered at the amide and carboxylic acid groupings of glutamine*

dl-Glutamic acid imide. α -Oximinoglutamic acid imide (see under: 2 g.) in water (20 ml.) and HCl (1.5 ml. conc.) was shaken with palladium charcoal (0.5 g.) in H_2 at slightly above atmospheric pressure. Gas was rapidly absorbed until 580 ml. had reacted (6 hr.) when the solution was filtered from catalyst and evaporated in vacuum at 45° to crystallization. Further material was obtained by the addition of alcohol and ether to the mother liquors. The combined products (M.P. 204 – 206°) were recrystallized from aqueous alcohol containing a little HCl, giving colourless prisms, M.P. 206° , of glutamic acid imide hydrochloride. Found: N, 17.15%. Calc. for $\text{C}_5\text{H}_9\text{O}_2\text{N}_2\text{Cl}$: N, 17.1%. The crude material exhibited small activity, which was lost on repeated crystallization.

dl-Isoglutamine was prepared from *dl*-glutamic acid according to Bergmann & Zervas' [1932] preparation of the *l*-acid. This is known to yield a mixture containing about 14% of glutamine [Melville, 1935]. When purified by heating the aqueous solution under conditions in which glutamine decomposes [Melville, 1935] it still contained labile amide (estimated by the procedure of Vickery *et al.* [1935]) corresponding to 1% of glutamine. Repetition of the process, and various crystallizations, reduced the glutamine to an amount not chemically detectable and at the same time gave greatly reduced growth activity. Though this was not completely removed the evidence obtained indicated it to be due only to traces of glutamine.

F. *Compounds altered at the amide, amine and carboxylic acid groupings of glutamine*

α -Oximinoglutamic acid imide. Diethyl- α -oximinoglutarate [McIlwain & Richardson, 1939] (8.8 g.) in a glass-stoppered flask, and saturated NH_4OH (20 ml.) were cooled in ice and mixed. The solid softened and two layers formed; it was shaken at room temperature until homogeneous (about 1 hr.) and kept for 6 hr. in all at room temperature. Periodical titrations of excess NH_3 in aliquot portions indicated the reaction to be complete at that time, when 0.205 mol. of NH_3 had reacted with 0.2 mol. of ester. The solution was evaporated in vacuum from a bath at 45° to about half its bulk, when crystals had begun to appear; it was cooled to 0° , the solid (5 g.) collected and recrystallized from water to colourless long or short prisms. The pure imide melted with decomposition to a green-blue liquid at temperatures between 155 and 165° according to the conditions of heating. Found: total N, 20.5%; amide N, 10.1%. Calc. for $\text{C}_5\text{H}_9\text{O}_3\text{N}_2$: 19.8 and 9.9%. The imide was soluble in water to a solution of pH 4–5. NH_3 was liberated by the action of cold conc. NaOH but the compound recrystallized unchanged from dilute HCl and was thus not an ammonium salt. It formed a precipitate with Hg^{++} salts in neutral, acid and alkaline solutions, but not with Pb or Ag salts.

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dl-Hydantoin-5-β-propionic acid was prepared by the action of HCl on *dl*-uramidoglutaric acid and formed colourless crystals m.p. 164–165°.

dl-Pyrrolidone-α-carboxylic acid amide was prepared from the acid according to Alberhalden & Kautzsch [1912] and repeatedly crystallized from aqueous acetone, yielding colourless prisms of the amide, m.p. 217°. Found: N, 21.5%. Calc. for $C_5H_8O_2N_2$: 21.85%.

G. Miscellaneous

Asparagine and *insulin* were commercial specimens.

DISCUSSION

Thus no growth occurred when practically any of the above compounds was substituted for glutamine in conditions under which glutamine itself caused growth. The immediate interpretation of these results is that the above compounds cannot perform the functions of glutamine, which are thus seen to be extremely specific.

There is also the further implication that none of the above compounds can be converted into glutamine by the organism at a rate sufficient to meet its demands in even extremely slow growth. Thus *Streptococcus* is seen to be incapable of such relatively simple operations as forming the γ -amide of glutamic acid, hydrolysing an *N*-acetyl, *N*-leucyl or *N*-cysteyl grouping; hydrolysing peptide linkages between the carboxylic acid grouping of glutamine and the amino groupings of glycine, glutamic acid or cysteine. It also cannot open the rings of pyrrolidone- α -carboxylic acid or glutamic acid imide by addition of NH_3 or water in the necessary positions.

These results can be considered in relation to the mode of action of glutamine. Particularly significant in this connexion is the inactivity of the glutamine peptides. It may be argued that if *Streptococcus haemolyticus* has no enzyme mechanisms capable of breaking down simple peptides to release glutamine, then, from the normal reversibility of enzyme systems, it is also incapable of building up glutamine into normal peptides. Thus the purpose for which glutamine is used by the organism is not peptide formation unless enzymes specific to a particular peptide are involved. The inability of *Streptococcus haemolyticus* to form unusual amide linkages has already been commented on [McIlwain, 1939]. With this reservation, it would appear that glutamine is used by the organism as such rather than for building up a larger molecule, as the compounds tested present examples of every likely type of combination with glutamine.

A suggestion as to the use of glutamine obvious from its peculiar properties is that of NH_3 transference. If glutamine is involved in such a cyclic process, the compounds into which it is converted would be expected to show glutamine activity. Nevertheless, ammonium pyrrolidone- α -carboxylate, the product of breakdown of glutamine in aqueous solution, is inactive, as also is glutamic acid, the product of action of glutaminase on glutamine.

SUMMARY

A large variety of glutamine analogues and derivatives have been found incapable of replacing glutamine in supporting growth of *Streptococcus haemolyticus*. The mode of action of glutamine is discussed.

I have pleasure in thanking Prof. A. C. Chibnall, Prof. C. R. Harington and Dr R. L. M. Synge for gifts of glutamine derivatives.

ADDENDUM

Note on the stability of glutamine. Data on the stability of glutamine in simple aqueous solutions have been recorded by Melville [1935] and by Vickery *et al.* [1935]. The following are points of practical importance which may be taken as supplementing those observations. (a) Solid glutamine prepared from beet and containing a little moisture was found to have decomposed to the extent of 66 % (as judged by labile amide-N) in the course of keeping for 3 months at room temperature. Later specimens have been kept over CaCl_2 at 0° . (b) In certain bacteriological culture media containing agar, glutamine was stable to autoclaving at 120° and pH 7.2 for 20 min.; $1.5 \times 10^{-5} M$ glutamine in one medium supported growth equally before and after such autoclaving. In peptone infusion not containing agar, some destruction of glutamine occurred (P. Fildes, G. P. Gladstone and G. M. Hills).

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CCXLII. THE ROLE OF SORBITOL IN THE C-METABOLISM OF THE KELSEY PLUM

II. RELATION OF CARBOHYDRATE AND ACID LOSS TO CO₂ PRODUCTION IN STORED FRUIT

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(Received 17 October 1939)

It was shown in a previous communication [Donen, 1939] that, in the Kelsey plum sugars, acid and sorbitol were the most likely sources of C in the respiration of the detached fruit, and that their combined loss accounted satisfactorily for the depletion of dry weight during storage. In this paper an attempt is made to construct a balance sheet relating C lost by stored plums as CO₂ with the C lost as sugar, acid and sorbitol. Such a balance sheet is of some interest, as little information is available of the extent to which compounds other than sugars, or known precursors of sugars, contribute towards the C lost as CO₂ by respiring plant tissue. In the leaves of barley [Yemm, 1935], rhubarb [Vickery & Pucher, 1939], and wheat [Krotkov, 1939] as well as in germinating rice seedlings [Dastur & Desai, 1935] the loss of carbohydrates alone is insufficient to account for the output of CO₂. Proteins or some undetermined carbohydrates have been suggested as the possible sources of the excess CO₂. In the apple the loss of acid and sugar was found to be 17–30% greater than the C given off as CO₂ [Archbold & Barter, 1934]. Although the metabolisms of the apple and the leaf are possibly not directly comparable, nevertheless this result is the direct opposite of what has been found for detached leaves. In the plum the C lost as CO₂ can be, under certain conditions, almost completely accounted for in terms of losses of sorbitol, acid and sugar, but it will be shown that this conclusion may be greatly modified by the length and storage conditions of the experiment.

EXPERIMENTAL

The data given in this paper are presented in the form of graphs. They deal with rates of loss of C in stored fruit and are based on results of experiments described in Part I of this study [Donen, 1939]. For details of analysis and of chemical change in stored Kelsey plums that communication should be consulted. Here only a brief outline will be given of the scheme of the experiments.

Five different sets of plums were taken for storage. The first three sets (S 1, S 2, S 3) were stored at 13° immediately on picking and represented fruit of different maturity and different chemical composition. S 1 consisted of young fruit containing 4% total sugar, 2% acid as malic and only 0.5% sorbitol. S 2 plums were picked midway in the growth cycle of the fruit and contained 7.3% sugar, 1.6% acid and 1.5% sorbitol. The S 3 group of plums represented mature fruit and contained 10% sugar, 1% acid and 2.5% sorbitol.

The other two sets of plums (S 4 and S 5) consisted of mature plums which were first stored for 21 days at 1° and then ripened at higher temperatures

(7.5 and 20°). S 4 consisted of fruit high in sorbitol and was identical in composition with S 3. In contrast to S 4, S 5 contained relatively little sorbitol (1.4 % in S 5 as compared with 2.5 % in S 4), but the sugar (9.3 %) and acid (1 %) contents were almost the same as in S 4.

The fruit was stored at the requisite temperatures immediately on picking, and the changes in sorbitol, sugar and acid were followed by analyses at 5 to 7 days' intervals. Curves of best fit were then calculated to the experimental points and from these curves corrected values of the estimates were obtained. These corrected values were used for the estimation of average rates of loss shown in Figs. 1-5. Differences between the observed and calculated values were used for the estimation of standard error. Significant differences in estimation of loss of C were then calculated and are shown alongside the appropriate curves.

CO₂ estimation was done on samples chosen from each different set of fruit and stored under the same temperature conditions. The CO₂ drift of S 1 was estimated on a sample of 32 plums. Such a large sample was not found suitable for the more mature series and the number was reduced to 24 for S 2, to 5 for S 3 and S 4, and to 8 for S 5. In selecting the smaller samples for CO₂ estimation, care was taken to choose plums which represented as nearly as possible the average of the population used for the experiment.

The arrangement of the apparatus for CO₂ estimation was briefly as follows. Air was blown by means of a pump through two bubblers containing strong NaOH and then through a trap containing soda-lime to absorb all traces of CO₂. The air stream was then passed through a water trap, through a bubbler containing CaCl₂ solution to adjust humidity and finally into a gas-tight container holding the fruit. The CaCl₂ bubbler and the water-trap were kept inside the constant temperature chamber to allow adjustment of the incoming air to the temperature of the fruit. The water-trap was essential to allow condensation of moisture in the incoming air as its temperature changed from 20° outside the chamber to 13° or 1° inside the chamber.

The CO₂ evolved was absorbed in a series of Pettenkoffer tubes which contained *N*/10 Ba(OH)₂ and were worked by a clock mechanism to allow automatic change of absorption tubes every 3 or 6 hr. Smooth curves were drawn through the CO₂ record thus obtained and the values given in the graphs are readings taken from these smoothed curves.

All results have been expressed in terms of C and the given rates of loss refer to mg. C lost per day per 100 g. of original fresh weight.

Results

Series I. Young plums contain little sorbitol, and in S 1 sugar and acid accounted for 80 % of the total loss of C estimated as sugar + acid + sorbitol (to be referred to as S.A.S.-C). Sugar and acid contributed C in almost equal proportions and the rates of loss of all three components were quite uniform throughout the observed storage period. In Fig. 1 the rates of loss of sugar and acid are represented as continuous straight lines. The fluctuations in the estimations of average rates that were actually obtained may be judged from the lines for total C.

The CO₂ curve showed a rapid fall during the first 7 days of the experiment and only during this period was the rate of loss of CO₂-C significantly higher than the observed rate of loss of S.A.S.-C. During the remaining period, including the slight climacteric rise towards the end, the loss of CO₂-C did not differ significantly from that of S.A.S.-C.

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After 64 days, when the experiment was terminated, the estimated loss of S.A.S.-C was 730 mg. (with a probable error of ± 41 mg.) compared with a CO_2 loss of 721 mg.

— S1—

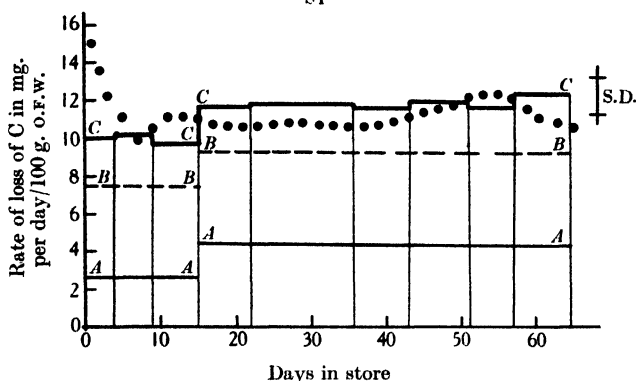


Fig. 1. Rate of loss of C in "young" plums (S 1) stored at 13° . AA, acid. BB, acid + sugar. CC, acid + sugar + sorbitol. Black circles, CO_2 .

— S2—

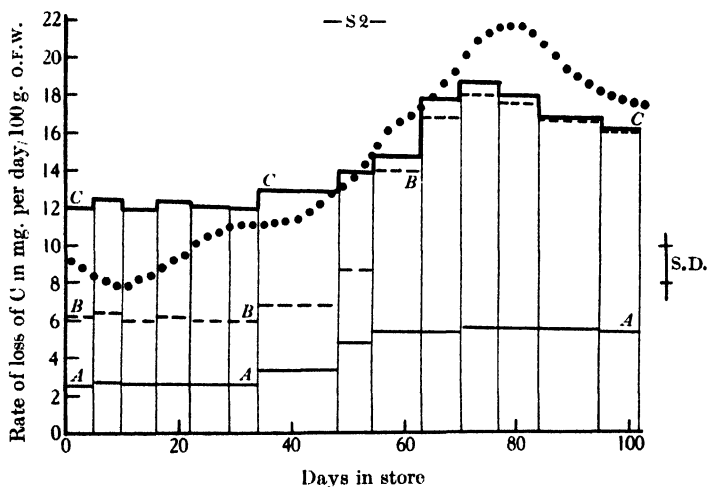


Fig. 2. Rate of loss of C in half-matured plums (S 2) stored at 13° . AA, acid. BB, acid + sugar. CC, acid + sugar + sorbitol. Black circles, CO_2 .

Series II. The experiment with the fruit of this series lasted for over 100 days. These plums contained a considerable amount of sorbitol and the loss of S.A.S.-C during the first 50 days was completely dominated by the rate of loss of the sugar-alcohol (Fig. 2). The latter contributed almost 50 % of the S.A.S.-C compared with a contribution of about 25 % each by sugar and acid. The CO_2 -C loss during the same time was significantly less than the S.A.S.-C loss and had the experiment been terminated after about 50 days it could have been concluded that the S.A.S.-C loss (594 ± 65 mg.) was 29 % greater than the CO_2 -C loss (458 mg.).

The sorbitol content of the plums in this experiment was almost completely exhausted during the first 50 days of storage, and its contribution to the

S.A.S.-C lost during the remaining period of the experiment was negligible. During the latter stage sugar loss accounted for 61 % of the S.A.S.-C and acid for about 33 %. This last stage was also marked by a rapid rise in the rate of CO_2 evolution which reached the peak of its climacteric on about the 80th day. It is of interest to note that the rate of loss of S.A.S.-C followed to some extent the rising curve of CO_2 -C, but even then the latter was significantly higher than the S.A.S.-C curve. During the last 48 days CO_2 -C loss was 13 % higher than the loss of S.A.S.-C. For the whole period of the experiment (102 days), however, C lost as sugar, acid and sorbitol almost exactly balanced the CO_2 -C. S.A.S.-C loss was 1490 ± 65 mg. CO_2 loss was 1470 mg.

Series III. The mature plums used for this experiment showed no significant change in sugar concentration when stored at 13° , and the C lost was due solely to loss of acid and sorbitol. The rate of acid loss was uniform throughout and it contributed only 17.8 % of the S.A.S.-C.

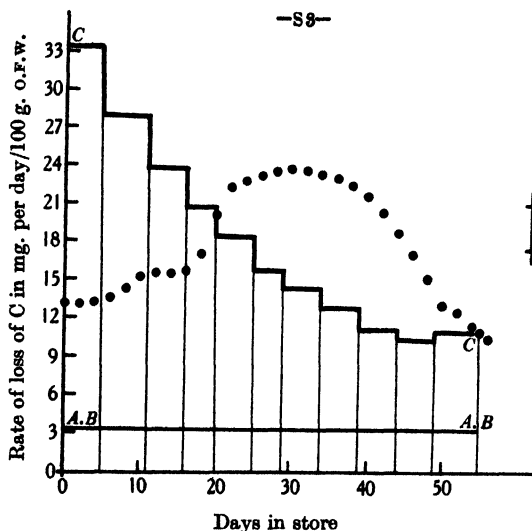


Fig. 3. Rate of loss of C in mature plums (S 3) stored at 13° . AA, acid. BB, acid + sugar. CC, acid + sugar + sorbitol. Black circles, CO_2 .

The rate of sorbitol loss followed an exponential curve; it was very high at the beginning but fell off rapidly towards the end of the experiment. During the first 20 days the loss of sorbitol-C alone was 57 % higher than the loss of CO_2 -C. If acid loss is included then during that period 78 % more C was lost as S.A.S. than as CO_2 .

The CO_2 curve rose steeply after the 16th day (Fig. 3) and reached the peak of its climacteric 15 days later. During the climacteric period (20th to 55th day) the CO_2 -C loss was far in excess of the loss of S.A.S.-C. The latter was 465 ± 65 mg. whilst the CO_2 evolved amounted to 696 mg. C, i.e. very nearly 50 % higher than the S.A.S.-C loss. This surplus of CO_2 -C was almost exactly balanced by the deficit during the first 20 days of storage so that for the whole duration (55 days) the CO_2 -C loss was 1000 mg. and that for S.A.S.-C 996 ± 65 mg.

It is apparent from the results so far presented that the losses of C measured as loss of sugar, acid and sorbitol were not a true measure of the actual amounts of C eliminated from the plum at all stages of the experiment. The fact that

apparent deficits of C at one stage were balanced by apparent surplus of C at another stage strongly suggests that sorbitol, and possibly even sugar, gave rise to intermediate breakdown products which were not measured by the analytical methods employed, but which were subsequently drawn upon in respiration, thus finally giving rise to CO_2 .

Series IV and V. These experiments were in essence studies of the extent to which the metabolism of Kelsey plums is modified by exposure to low temperatures (1°), and how this is again affected by transferring the fruit back to a higher temperature (20 or 7.5°). The complex series of changes that occur in the rates of loss of sugar, acid and sorbitol are shown in Fig. 4 (for S 4) and in Fig. 5 (for S 5).

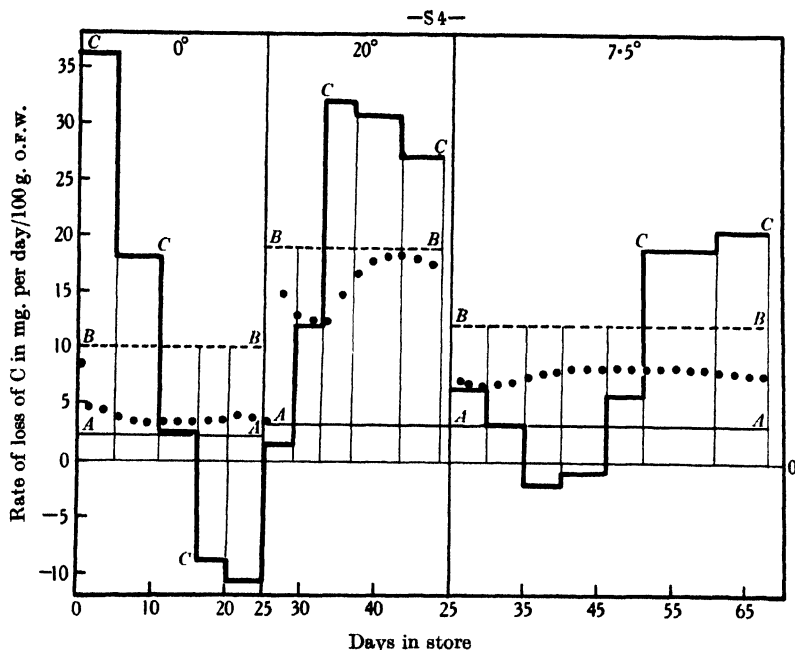


Fig. 4. Rate of loss of C in mature plums (S 4) stored for 25 days at 1° and then transferred to 7.5 or 20° . AA, acid. BB, acid + sorbitol. CC, acid + sorbitol + sugar. Black circles, CO_2 . For further explanation see text.

In both graphs the AA line above the zero line, 00, represents rate of loss of acid. The BB line indicates the rate of loss of acid and sorbitol combined, and the CC line that of acid + sorbitol + sugar. The rates of loss of sugar are measured as the distances along the ordinates between BB and CC, and are positive if CC is above BB, and negative (i.e. there is a gain in sugar) if CC is below the line BB.

Reference has already been made [Donen, 1939] to the fact that exposure of the fruit to 1 or 0° introduces effects which are not merely the direct result of retardation of rates of change by low temperature. In Fig. 4 it is shown that an initial loss of sugar in S 4 on storage at 1° was reversed to a gain after 10 days, and that this increase in sugar persisted not only during the remaining period of storage at 1° but also for a considerable time after the fruit was transferred to 20 or 7.5° .

The considerable loss of sugar shown in S 4 during the first 10 days is of interest, for during that period the loss of sorbitol and acid was more than

sufficient to account for the loss of CO_2 -C. S 5 did not show this initial loss of sugar. In another experiment with Kelsey plums, and also in several experiments with Peregrine peaches, it was found, however, that such sugar depletion is the usual occurrence during the first 10–12 days of storage at 1 or 0°. Its possible significance will be referred to in a later section of the paper.

The increase of sugar in cold-stored plums was observed in both S 4 and S 5, as well as in other Kelsey plums similarly treated. It was not observed in plums stored at 13° or at higher temperatures and must be, therefore, an effect peculiar to low temperature storage.

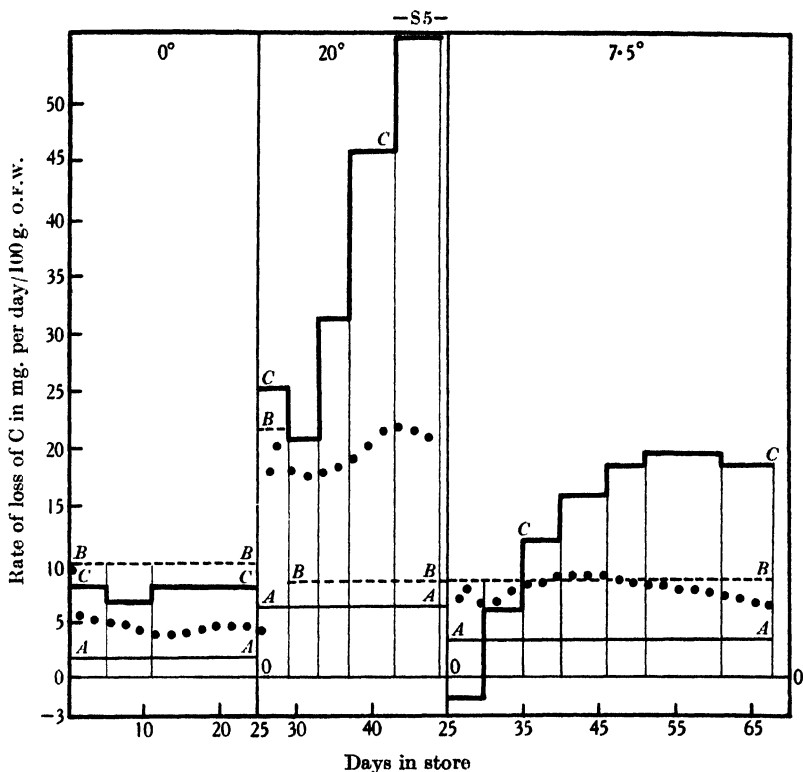


Fig. 5. Rate of loss of C in mature plums (S 5) stored for 25 days at 1° and then transferred to 7.5 or 20°. AA, acid. BB, acid + sorbitol. CC, acid + sorbitol + sugar. Black circles, CO_2 . For further explanation see text.

S 4 plums showed a more pronounced sugar synthesis than S 5, and in both series post-storage at 7.5° was more effective than 20° in prolonging the period of this sugar increase.

The source of C for the synthesis of sugar is of some interest here. In Table I an analysis is given of the amounts of C gained or lost by the various constituents at the moment of maximum increase in sugar in the two sets of plums. The loss of C as acid and sorbitol was sufficient to account both for the loss of CO_2 and for the gain in sugar. But it will be observed that although some of the C for sugar formation might have arisen from acids, even the total C lost as acid was not sufficient to account for all the increase in sugar and a good proportion of the latter must have had sorbitol as its original source. In each case, moreover,

Table I

C-balance at points of maximum rate of sugar increase. All results in mg. C.

	Period	Net sugar gain	Loss of sorbitol	Loss of acid	Loss of S.A.S.-C	Loss of CO ₂ -C	Excess S.A.S.-C over CO ₂ -C %
S 4	25 days at 0° and 8 days at 20°	138	324	82	268	198	35
	25 days at 0° and 26 days at 7.5°	258	427	139	308	294	5
S 5	25 days at 0°	60	205	42	187	114	64
	25 days at 0° and 10 days at 7.5°	106	258	74	226	181	25

there is (considering the whole period) sufficient C from sorbitol breakdown to account for all the increase in sugar, and this, coupled with the observation that low sorbitol fruit (S 5) showed a much less pronounced sugar increase than plums having much sorbitol (S 4), points to the probability of sorbitol being the sole source of synthesized sugar in Kelsey plums stored at low temperatures.

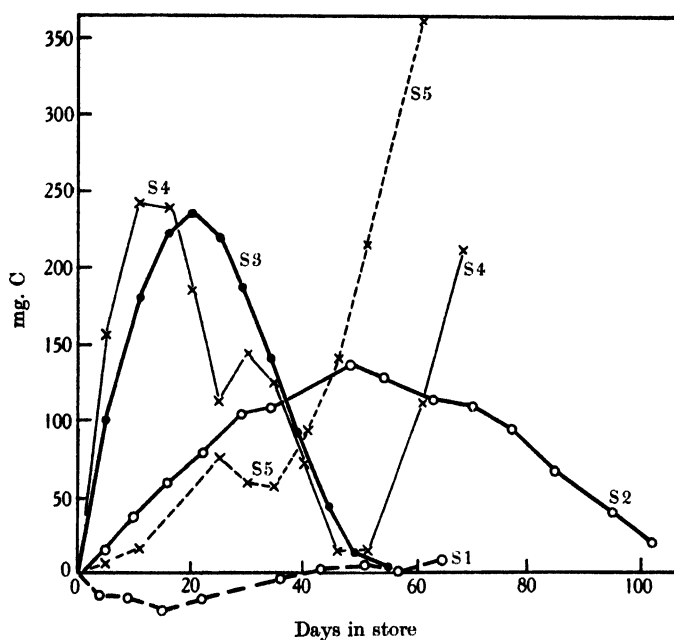


Fig. 6. The variation in excess of C lost as sum of acid, sugar and sorbitol over C lost as CO₂ with time in store. S 1, S 2 and S 3 stored at 13°. S 4 and S 5 stored for 25 days at 1° and then at 7.5°.

Throughout the duration of the S 4 and S 5 experiments the accumulated loss of S.A.S.-C was in considerable excess over the total loss of CO₂-C. As in S 2 and S 3 there were short periods, however, over which the loss of C as CO₂ was in excess of S.A.S.-C loss, thus providing further evidence of accumulation of intermediate breakdown products of sorbitol and sugar. During the period of maximum sugar increase the concurrent loss of sorbitol could not possibly have accounted for the gain in sugar, and intermediate compounds resulting from

previous breakdown of sorbitol must therefore have been utilized in its synthesis. The variation in the amounts of these undetermined breakdown products is shown in Fig. 6. In S 4 and S 5 there was an initial accumulation of undetermined products which were depleted during the stage of sugar synthesis and reached a minimum at the point of maximum sugar concentration.

The rapid breakdown of sugars, observed towards the end of the experiment in both series of plums, is rather curious and not compatible with any theory of mere utilization of carbohydrate for respiration. The transition of sugar synthesis to sugar breakdown occurred at a stage when there was still a considerable amount of C unaccounted for as CO_2 and at a time when, for instance in S 4, sorbitol concentration (1 %) and its rate of loss were still quite high (Fig. 4). When the experiments were terminated the two sets of S 4 plums stored at 20 and 7.5° showed an excess of S.A.S.-C over CO_2 -C equal to 54 and 50 % respectively. The corresponding figures for S 5 were 106 and 100 %. These differences were most marked during the period immediately following the maximum sugar increase. In S 5 plums, post-stored at 20°, S.A.S. loss was 733 mg. C per 100 g. of original fresh weight over a period of 16 days. Sugars contributed 604 mg. of that. The CO_2 lost during the same time amounted to only 295 mg. C.

DISCUSSION

From the foregoing results it is clear that in considering the relationship between respiratory CO_2 and the substrates that give rise to it, low temperature effects should be allowed for. The storage temperature of 13° was chosen for the present experiments because it was known that 13° was outside the range of temperatures at which low temperature breakdown had previously been observed. Parallel experiments at 25° gave results similar to those at 13° and the results at the latter temperature may therefore be assumed to be representative of the normal metabolism of the Kelsey plum.

Maturity of the fruit, with the corresponding variation in sorbitol content, appears to be the most important factor governing the C-balance of the Kelsey plum at high temperatures. In young fruit containing little sorbitol CO_2 -C losses balance quite accurately the losses of acid, sugar and sorbitol at almost any stage of the experiment (e.g. S 1, and see Table II). As the fruit matures and its sorbitol content increases, a C balance may still be obtained but such agreement is, in a sense, accidental, as it depends entirely upon the length of the experiment and on the previous history of the fruit (see S 2 and S 3 and Table II). This is illustrated by the curves shown in Fig. 6 which trace the excess of C lost as

Table II. *Maturity and loss of sugar, acid and sorbitol*

The weight of the fruit in the first column is given as a guide to the maturity of the Kelsey plums on picking. They were then stored for 26 days at 25°. The given losses of C represent total loss after 26 days at 25°.

Wt. per fruit in sample g.	Initial sorbitol content % o.f.w.	Sugar lost in mg. C	Acid lost in mg. C	Sorbitol lost in mg. C	Total C loss S.A.S.-C	Loss of CO_2 -C
24	1.25	368	92	238	698	650
43	0.97	284	32	87	403	429
73	1.31	192	68	202	462	420
93	1.99	288	122	364	774	431
137	2.71	4	86	412	502	382
138	2.66	0	112	556	668	452

sugar, acid and sorbitol over CO_2 -C liberated by the fruit during the course of the various experiments.

The results shown in these curves are readily explained on the hypothesis that when sorbitol is broken down not all of its C is immediately utilized for CO_2 formation, but that some of its breakdown products are accumulated in the fruit. This might happen if the rate of sorbitol breakdown is greater than the rate of CO_2 formation from the intermediate products, or, if sorbitol gives rise to more than one type of intermediate compound and one of these is preferentially respired. Accumulation of intermediate C compounds not recorded either as acid, sugars or sorbitol then takes place and continues until the gradual depletion of sorbitol results in a slowing down of the breakdown of sorbitol to a rate below that of CO_2 formation. The accumulated intermediate C compounds are then rapidly utilized and finally exhausted. If at the same time the sorbitol level becomes very low, more sugar is utilized (as in S 2), but if rate of loss of sorbitol is still considerable, sugars are not used (S 3).

The relation between sorbitol and sugar utilization is not quite clear but possibly the two processes are distinct. It is probable that sorbitol breakdown is an irreversible process, as no increase in sorbitol has been observed in any of the experiments with stored plums. Also, at high temperatures sorbitol either does not give rise to sugars or does so at a very slow rate, for otherwise an increase in sugar should have occurred in S 3 where loss of sorbitol was far in excess of the CO_2 requirements during the first 20 days in store.

It is generally accepted that low temperature may profoundly affect the metabolism of detached plant organs and Barker's work on potatoes [1933] suggests that such effects might last for some considerable time after the return of the organ to higher temperatures. In the plum, low temperature storage affects both the sugar and sorbitol metabolism and results in a series of changes which appear successively as sugar loss, sugar synthesis and finally sugar breakdown once again.

More experimental evidence is needed to clarify this complex process, but a working hypothesis may be ventured here. The observations described above may be pictured as the resultant of two simultaneous processes induced in the fruit by low temperature. The first is a stimulation of sugar breakdown: and this stimulation persists for a considerable time after the return of the fruit to higher temperatures. Such a process would explain the rapid initial sugar breakdown at low temperature as well as the loss of sugar during post-storage at the high temperatures. Superimposed upon the first process is a second one resulting in stimulation of sugar synthesis from sorbitol; it only occurs at low temperatures but its maximum rate is higher than that of the first process of sugar breakdown. When the fruit is returned to high temperatures sugar synthesis from sorbitol is immediately slowed down, more rapidly by a temperature of 20° than by that of 7.5° . This second process would account for the observed change over from initial sugar breakdown to sugar synthesis at low temperature and for the reversal of that on returning the fruit to the higher temperatures. It fits in with the observations that sugar increase ceased in S 4 during post-storage when ample supply of sorbitol was still available and that it did not occur at all in fruit which was not first cold-stored.

It is of interest here that the results obtained with S 4 and S 5 suggests that, like sorbitol, sugars are also capable of giving rise to considerable quantities of intermediate C compounds which are not completely utilized in respiration. This conclusion is in no way incompatible with modern ideas on respiration, but the possible accumulation of such large amounts of breakdown products of sugar has not been previously reported.

To what extent similar sugar breakdown occurs in other plant organs stored at low temperatures is not known, but the results obtained with the Kelsey plum offer an explanation of the findings of Archbold & Barter [1934]. These workers stored their apples for some considerable time at 1° and then used them for respiration tests at 12°. Their observation that more C was lost as sugar and acid than was accounted for by $\text{CO}_2\text{-C}$ is similar to our observations on Kelsey plums stored at low temperature. It is quite possible that they would have obtained different results if their experimental material had not been cold-stored prior to the beginning of their experiment.

In view of the important part played by sorbitol in the C-metabolism of the Kelsey plum it is essential to investigate what role hexitols in general play in the metabolism of higher plants. Strain [1937] and Reif [1934] have indicated that sorbitol, and probably other higher alcohols, are present in a great number of leaves and fruits. The ordinary methods of sugar analysis do not account for hexitols, and some of the sources of C in respiration which have been ascribed to unknown carbohydrates or to proteins might well have had their origin in compounds of this type.

SUMMARY

A comparison has been made of the C lost as sorbitol, sugar and acid (S.A.S.-C) with the C given off as CO_2 by Kelsey plums stored at 13°.

It is shown that maturity of the fruit, with the corresponding variation in sorbitol content, is the most important factor governing the C-balance in plums stored at high temperatures. In young plums, containing little sorbitol, the loss of S.A.S.-C balanced quite accurately the loss of $\text{CO}_2\text{-C}$ at almost any stage of the experiment. In mature fruit this balance could also be obtained, but agreement between losses of S.A.S.-C and $\text{CO}_2\text{-C}$ was accidental and depended entirely upon the length of the experiment.

The results show that loss of S.A.S.-C was not a true measure of the actual amount of C eliminated from the plum at all stages of the experiment and it is suggested that sorbitol and sugars gave rise to intermediate breakdown products which could subsequently be drawn upon in respiration and thus finally gave rise to CO_2 .

When plums were stored at 1° and then returned to higher temperatures (7.5 and 20°), effects were induced which profoundly affected both the sugar and sorbitol metabolism of the fruit. A series of changes resulted which appeared successively as sugar loss, sugar synthesis and finally sugar breakdown once again. It is suggested that these changes were the resultant of two simultaneous processes induced in the fruit by low temperature and consisted of sugar breakdown and concurrent sugar synthesis from sorbitol. The S.A.S.-C loss in such plums was always much greater than the corresponding $\text{CO}_2\text{-C}$ loss.

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CCXLIII. NITROGEN EXCRETION AND ARGINASE ACTIVITY DURING AMPHIBIAN DEVELOPMENT

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MUCH evidence has accumulated to show that ammonia and urea excretion is characteristic of aquatic and amphibian life, and uric acid of terrestrial life [Needham, 1930; Delauney, 1935]. An ontogenetic sequence similar to the above has been shown to occur during the development of the chick [Needham, 1925]. During ontogenesis, at one stage 90% of the N excretion is present as NH_3 . On the other hand, examination of recent data for partition of urinary N, compiled by Needham [1930], shows that in no aquatic animal investigated is a purely ammoniacal urine excreted.

Clementi [1914] has pointed out that the enzyme arginase is present in the livers of ureotelic animals, but absent where the metabolism is uricotelic, while Krebs & Henseleit [1932] have shown how this enzyme fits into a cyclic mechanism for urica production by the liver. But the factors controlling the balance of NH_3 and urea in the excretion of vertebrates other than mammals have not received much attention.

The questions arise: are there certain aquatic types amongst the vertebrates whose excretion is neither predominantly ureotelic nor uricotelic but almost exclusively ammoniacal? Are there specific environmental conditions causing the excretion of a predominantly ammoniacal urine? Is arginase also absent from the livers of those types? It was believed that a determination of the NH_3 and urea production during development and metamorphosis of the frog might be illuminating.

In this paper data are given relating to the N partition, the intensity of N excretion under various experimental conditions, and the arginase activity of the liver at various stages in the development of *Rana temporaria*.

Material and methods

Tadpoles were collected late in March from a loch in the neighbourhood. They were at the stage where external gills were just disappearing. Batches of approximately 500–1000 were found densely crowded in isolated pools in the weed fringing the water. From the uniformity of size and development it was assumed that each group had developed from a single batch of eggs. During development they were fed on ant pupae and kept at about 17°.

For the estimation of excreted NH_3 and urea, batches of 5–50 tadpoles, according to the size of the individuals and the duration of the experiments, were kept in evaporating basins, in volumes of water varying from 15 to 50 ml. Some of the first experiments were done with the tadpoles immersed in tap water or distilled water, but it was believed that excreted NH_3 might be lost from such fluid and all later experiments were done with fluid very lightly buffered with

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phosphate at pH 6.5. Estimations were also made of free NH_3 and urea- NH_3 in the excretion of tadpoles kept at five different pH ranging from 6 to 8. The ratio of free NH_3 to total NH_3 was found to be the same at each pH, viz. 92 : 100.

Ammonia was determined by the Conway-Byrne [1933] diffusion method. As the ordinary Conway unit was too small for the volumes of fluid here determined, pyrex petri dishes of 4 in. diameter with ground glass rims were used. In the centre of each was placed a shallow watch glass to hold 1 ml. 0.01 N HCl. The unit was sealed by means of a glass plate smeared with vaseline. Ammonia was liberated by saturated K_2CO_3 solution. The unit was left for 18 hr. to allow diffusion of the NH_3 into the standard acid, which was subsequently titrated with 0.0025 N NaOH, using Tashiro's indicator. Repeated estimations with standard solutions of NH_4Cl , with concentrations of NH_3 in the units varying between 0.017 and 0.136 mg., gave results of an accuracy not less than 95%. The same method was used for the estimation of excreted urea. A similar degree of accuracy was obtained when urease, freed as far as possible of NH_3 by shaking with permutit, was used. Urea was estimated in the arginase experiments by the manometric method of Krebs & Henseleit [1932].

For convenience in presentation of the Tables, the development has been divided into a number of arbitrary stages as follows: I. Hindlimbs absent. II. Hindlimbs just visible. III. Hindlimbs $\frac{3}{4}$ grown. IV. Hindlimbs almost fully grown and freely movable. V. Hindlimbs fully grown. Forelimbs fully grown but still covered. Intestine whitish and actively shortening. VI. One forelimb emerged. VII. Both forelimbs emerged. Tail still fully developed. VIII. Tail atrophying. IX. Tailless.

EXPERIMENTAL

Table I shows the effects of starvation, larval weight and developmental stage on the N excretion. The NH_3 and urea excretions calculated per g. wet

Table I. *Effects of size and starvation on N excretion of developing frogs. Temperature 17.5°*

	Average individual weight in mg.	Developmental stage	ml. 0.0025 N NaOH		Total output per g. animal per 24 hr.	% as free NH_3
			Free NH_3	Urea NH_3		
Direct from food; exp. over 20 hr.	310	II-III	3.10	0.50	5.3	86
	315	IV	2.30	0.51	5.4	82
	300	V	1.45	0.69	5.3	67
	176	VIII	0.52	2.10	8.0	25
Starved 24 hr.; exp. over 8 hr.	30	I	0.73	0.08	5.5	90
	56	I	1.19	0.13	5.7	90
	104	II	2.02	0.27	5.4	88
	210	III	1.59	0.21	4.6	81
Starved 48 hr.; exp. over 20 hr.	40	I	1.48	0.15	4.9	91
	75	I	1.15	0.19	3.3	85
	250	III	1.49	0.16	3.5	90
	210	V	1.25	0.69	4.5	64
Free NH_3 + Urea NH_3						
Starved 72 hr.; exp. over 20 hr.	205	II-III	1.42		3.6	—
	200	IV	1.23		3.7	—
	194	V	0.36		2.6	—
Starved 96 hr.; exp. over 20 hr.	200	II-III	1.35		3.2	—
	190	IV-V	1.01		2.5	—

weight of animal are much the same in both small and young tadpoles as in the metamorphic stages. This is the case both in fed animals, growing and presumably still absorbing food, and in starved animals. Prolonged starvation, however, has the effect of reducing considerably the N output in all tadpole stages. The sudden increase in N output associated with stage VII, i.e. where both forelimbs have emerged, is very definite and amounts to an increase of at least 50%. It is not possible at present to give the reason for this sudden change in intensity of metabolism.

Table II. *Effect of temperature on ammonia and urea production of different developmental stages*

Temperature	Developmental stage		No. of animals	ml. 0.0025 N NaOH per g. animal per 24 hr.	% as NH_3
	Beginning	End			
4°	II-III	II-III	7	1.51	90
	IV	IV	5	1.49	74
	V	3 at V 1 at VI	4	1.60	68
	VII	VII	5	3.00	32
	VII-VIII	VII-VIII	4	8.5	19
17°	II-III	II-III	6	5.4	85
	IV	IV	5	5.9	83
	V	1 at V 1 at VI 2 at VII	4	5.9	64
	VII-VIII	VII-VIII	4	8.5	19
	VII-VIII	VII-VIII	4	8.5	19
28°	II-III	II-III	6	10.3	89
	V	4 at V 1 at VI	5	10.8	70
	VII-VIII	VII-VIII	5	—	—
	VII-VIII	No survival	5	—	—

The effect of rise in temperature is to increase markedly the excretion of NH_3 and urea (Table II). Again it is emphasized that at all stages at similar temperatures the output of NH_3 and urea per g. of larvae gives a total of the same value.

As shown by Fig. 1, the Q_{10} is about the same for all tadpole stages. Between 4° and 14° it has the value of 2.8 and between 14° and 24° of approximately 2. The curves are of the same type, slightly convex to the abscissa. There is also a suggestion that the N excretion at stage V is slightly greater at all temperatures than that at stage II-III.

Again a pronounced increase in N excretion associated with the development of stage VII is apparent at each temperature. The curve also approaches very near to a straight line. Data [Pryzlecki *et al.* 1922] for the total NH_3 and urea excretion by the adult frog are also shown in the graph. It is noticeable that the points for froglet and adult frog form practically straight lines running almost parallel with one another, that for the froglet being at a slightly higher level. The temperature characteristics as regards N output are therefore different in the tadpole and frog.

There was no survival of froglets kept at 25° and 28° for 24 hr. The highest survival temperature over 24 hr. was 21°. This is in striking contrast to the experiments on tadpoles which are still healthy after 24 hr. at 28° and on adult frogs (Pryzlecki) where survival was obtained after 24 hr. at 31°.

Estimation of the N excretion of tadpoles at stage I gave the rather surprising result, that of the total amount of NH_3 liberated by treatment with

urease and K_2CO_3 , at least 90% was present as free ammonia and 10% was combined as urea. It was at first thought that this very high NH_3 content might be due to bacterial decomposition of urea occurring during the experiment. After an experimental period of 20 hr. a good deal of excrement had undoubtedly been shed into the fluid. To test this, two experimental dishes were set up, each containing the same number of tadpoles at a similar developmental stage. To the fluid in one dish was added a small concentration of urea. After an experimental period of 20 hr. estimation of free NH_3 in both dishes gave similar values. This experiment also ruled out the possibility of decomposition of urea by K_2CO_3 during the relatively prolonged estimation period.

In a second experiment tadpoles were starved over a period of 6 days, new fluid being put in each day, and immediately after the experimental period analysed for NH_3 and urea. On continued starvation excrement ceases to appear in the fluid, yet even on the sixth day of starvation free NH_3 still formed 90% of the total.

Experiments similar to the above were done on adult frogs, with which a free NH_3 percentage in no case higher than 12% was obtained. The experiments summarized in Table III were done with the object of determining at what stage

Table III

Developmental stage	Duration of stage	% present as free ammonia
I	60 days	92
II	15 "	84
III	15 "	84
IV	5 "	—
V	5 "	68
VI	10-20 hr.	—
VII	1-2 days	54
VII-VIII	1 day	29
VIII	1 "	26
IX	—	12

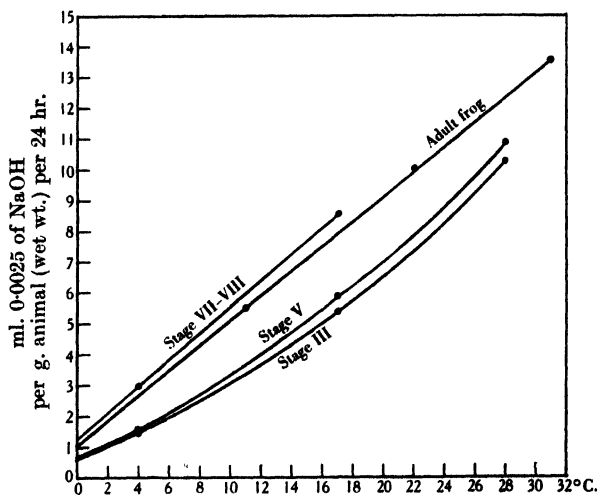


Fig. 1. Effect of temperature on the combined NH_3 and urea production at different stages of development.

or stages in development the change to predominantly urea excretion occurred, whether it was a gradual change over or an abrupt transition. It must be noted

that in spite of the use of tadpoles from the same batch, a very great variation occurred in the time required for individual animals to attain particular developmental stages. This applies more particularly up to stage IV. There are also great variations in the individual weight of animals from the same batch at similar stages and it is invariably found that a weight loss of almost 50 % occurs at metamorphosis. Accordingly, the durations of stages given in the table are necessarily approximations.

The results show that NH_3 production forms rather above 90 % of the N output in animals about 40 days old. The value falls slowly during the next month to about 85 % and during the next 10 days, at a period concomitant with rapid growth of the forelimbs and shortening of the intestine, to 68 %. During the next 4 days and beginning during the 10–20 hr. associated with emergence of both forelimbs there is a decrease in NH_3 of about 40 %, and a correspondingly great increase in urea production. During the next 3 days and paralleling the atrophy of the tail there is a rather slower decrease in NH_3 from 29 to 12 %, which is approximately the adult level.

Arginase activity of liver tissue during development

Livers were dissected out under a binocular microscope. To prevent any disturbing factor due to inactivation of arginase in the tissues during preparation a rotational procedure was adopted for the dissections. Liver was removed from one animal at stage III, one animal at stage V, one at stage VII, then back to the first stage. Tissue immediately after dissection was dropped into ice-cold frog Ringer solution. Dissection of 20 animals occupied 20 min. The combined livers for each stage were then placed on filter paper to remove excess water and quickly weighed. Arginase was extracted by grinding with quartz sand in a small volume of distilled water [Baldwin, 1935] for 5 min. and making the volume in each case up to 8 ml. Centrifuging for 5 min. brought down the sand and organic debris and a fairly clear yellowish fluid was obtained. 3 ml. of the fluid from each stage were added to boiling tubes containing 1 ml. glycine buffer of pH 9.8 and 1 ml. of a solution containing 40 mg. pure arginine. Adequate controls of brei and chemicals for urea content were made as shown below in the protocol for one experiment. The tubes were placed in a thermostat at 28° for 90 min.

Table IV

Exp. no.	Developmental stage	Liver wt. g.	No. of animals	Total body wt. g.	Liver wt. : body wt.
I	III	0.0091	8	0.982	1 : 100
	V	0.0412	6	1.393	1 : 30
	VII	0.0280	6	0.903	1 : 30

Table V

Exp. no.	Manometer no.	1	2	3	4	5	6	7
I	Manometer contents	Brei III + arginine	Temp. control	Arginine	Brei VII	Brei V + arginine	Brei VII + arginine	Brei V
	ml. taken for estimat.	2	—	—	2	2	2	2
	$\mu\text{l. CO}_2$ evolved	+6	0	+1	+1.5	+42	+106	+1.5
	$\mu\text{l. urea-CO}_2$ per mg. liver	4.1	—	—	—	9.8	30	—

No suggestion is made that the figures express the true proportionality of the arginase activities of the tissues. It was thought sufficient to show whether or not an increased activity of the enzyme was apparent during progressive developmental stages. The urea production during incubation therefore is expressed as $\mu\text{l. CO}_2$ per mg. wet weight of tissue. Table VI shows that there is an increasing activity of enzyme, that the activity is very small in the early stages increasing only slowly up to stage V and that it increases very greatly during the short period of 2-3 days covering stages V-VII.

Table VI
 $\mu\text{l. urea-CO}_2$ at 28° per mg. wet weight of liver

Exp. no.	Stage III	Stage V	Stage VII
I	4.1	9.8	30
II	2.5	7.4	23.5
III	1.4	6.0	20.2

Actually there are some indications that the increase in arginase activity is even greater than is suggested by the figures given. An experiment was set up to determine the arginase concentration and activity relationship in froglet liver, during 90 min. at 28° . The curve obtained was at no part a straight line and fell off very rapidly at the higher concentrations which were just outside the range of the experiments quoted. Other experiments to determine the time/activity relationship showed a diminution in arginolytic activity before the end of the first hour at 28° . In order to determine the true proportionality of the increase it would be necessary to decrease greatly the incubation time as suggested by Baldwin [1935]. This would require the preparation of a greater amount of tissue in order to be sure of accuracy in estimation of the early stages, but would simultaneously involve a longer period of preparation.

DISCUSSION

Experiments on the nitrogen excretion of frog larvae have previously been done by Bialascewicz & Mincovna [1921]. They state that the N excretion is almost equally divided between urea and NH_3 in tadpoles up to 300 hr. old and this value is maintained from the earliest developmental stages. Unfortunately no experiments were done during the work here described on such early stages. The earliest used which just follow on loss of the external gills have a urea excretion of less than 10%. It will be necessary to repeat these early experiments since a N excretion fluctuating thus would be remarkable.

It is necessary to consider first why the tadpole excretes a predominantly ammoniacal urine. Needham [1930] suggests that "The great disadvantage of NH_3 excretion is that a constant supply of acid is required to neutralize it". That the larvae survive is justification for saying that NH_3 excretion is possible under the conditions. One of the main considerations is that the larvae are living in a medium whose osmotic pressure is far lower than that of their blood. Therefore water is being absorbed and a large urine flow necessarily ensues, which would both wash the NH_3 thoroughly out of the kidney and also keep the concentration low at all times. Yet it probably still holds that an equivalent of acid is required for the excreted NH_3 . It would be of importance to determine the nature of this acid in order to see whether it is really waste or valuable substance necessarily lost. Even if acid is lost it may be compensated for by the fact that the energy used in production of NH_3 is much smaller than that involved in urea formation, as pointed out by Needham [1930].

For comparison with the tadpole experiments, estimation was made of the N partition in the excretion of starved eels. Free NH_3 formed 90 % of the total NH_3 liberated on addition of urease and potassium carbonate. Manderschied [1933] did not find the ornithine cycle present in the livers of two fresh-water teleosts, from which it might be concluded that urea in the excreted fluid is minimal, and confined to that arising from purine breakdown. Experiments with surviving slices of liver from the eels used above showed neither arginase activity nor evidence of the ornithine cycle. These experiments suggest that the ornithine cycle and probably arginase is absent from the livers of fresh-water fishes, but more data must be collected before this can be concluded. On the other hand, as Hunter & Dauphinee [1924] have shown, arginase is present in the livers of all salt-water fishes so far investigated.

Taking into account these facts, it seems that, as a method of getting rid of waste N, NH_3 excretion is more efficient than urea excretion for the tadpole with its typically piscine characters; and also, even although the animal is in the larval state, the lack of arginase in the liver is not due to larval simplicity of structure, but is essentially an established state for fresh-water existence. This state is only upset when its efficiency is impaired by a new level in development being reached, viz. for land existence. That this is the case is suggested firstly by the relative abruptness of the transition, and secondly by the fact that the initiation of this change is coincident with the period when the gills are shrivelling and the forelimbs are just piercing the gill coverings, and thus giving rise to a functionally active terrestrial animal.

Looking at the above results as a whole, it is significant that there are changes occurring practically at the same time in

- (1) the ratio of excreted urea- NH_3 : free NH_3 ,
- (2) the total urea + NH_3 excreted,
- (3) the arginase activity of the liver,
- (4) the weight of the liver.

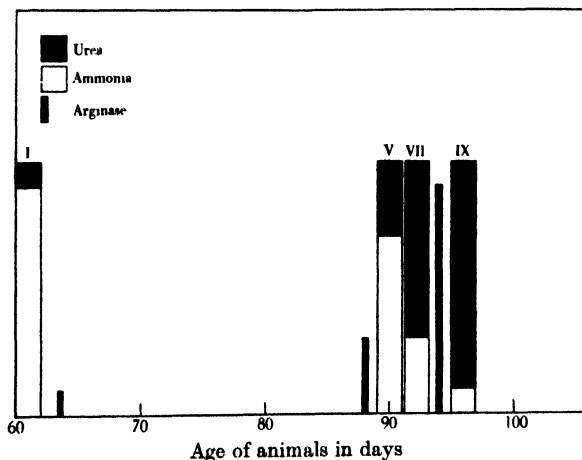


Fig. 2. The proportion of NH_3 to urea in the excretion at different stages during development. The relative arginase activity of the liver is shown at corresponding stages.

The histogram (Fig. 2) shows how the arginase activity although only expressed in the roughest proportionality corresponds strikingly with the

changing rate of urea excretion. The increase in urea is accompanied by a decrease in NH_3 and it can be inferred that the NH_3 which is disappearing is built up into urea. As no chemical mechanism other than the ornithine cycle, involving arginase activity, is known which can produce urea from NH_3 , it can also be inferred that this mechanism is being brought into play in a very short period of time.

The parallelism does not hold so well for the corresponding changes in liver weight and nitrogen output. The liver increases rapidly in size from a one-lobed to a three-lobed structure during the period preceding the rapid metamorphic changes associated with emergence of forelimbs, and it has already reached a maximum size before there is any great increase in apparent arginase activity. The increase in size, however, is interesting in that it must be associated with an increase in cell division as the extra lobes could not be formed otherwise. Whether the increase in cell number is related to increase in enzyme formation is open to speculation. Sohngen & Coolhaas [1924] have stated that galactozymase formation is accompanied by an increase in cell count.

Finally, one cannot but be impressed by the parallelism which exists between the degree of thyroid liberation at various stages and the changes listed above. The investigations of many workers have made it clear that "there is an accumulation of colloid during the early development of the hindlegs followed by active colloid discharge resulting in the rapid body changes of metamorphosis such as shrinkage of the gills, intestine and tail, and growth of forelimbs" [Allen, 1938]. It is possible that liberation of thyroid substance which has been proved to be the stimulus for the structural changes also exerts a direct effect on the N metabolism of the liver. Terroine & Bonnet [1936] find that injection of thyroxine into the pig increases the total N output. The fractions responsible for the increase are urea and creatine. Lanczos & Mansfeld [1939] find that injection of thyrotropic hormone into the adult frog increases the N metabolism. Edlbacher & Schuler [1932], on the other hand, tried the effect of thyroxine on mammalian arginase. Activity of the enzyme was found to be inhibited by about 30%. All these experiments suggest that one way or another thyroid has some effect on N metabolism.

It is intended to repeat the above experiments on axolotls fed with thyroid substance.

SUMMARY

The total of NH_3 and urea excretion in tadpoles has a similar value in small and large animals at all stages of development including the metamorphic period. The N output is markedly increased in the froglet and adult frog.

Rise of temperature increases greatly the NH_3 and urea excretion in all tadpole stages and in froglet and frog.

A rapid change over from predominantly NH_3 excretion to predominantly urea excretion is shown to occur during the metamorphic crisis.

An increase in liver arginase occurs at the same time as the above transformation, the most rapid and greatest increase being during active metamorphosis.

I wish to express my thanks to Prof. E. W. H. Cruickshank for his advice and criticism.

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CCXLIV. THE THIOCHROME TEST FOR ANEURIN (VITAMIN B₁) IN URINE AS AN INDEX OF NUTRITIONAL LEVEL

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THE method of Jansen [1936] for estimating aneurin by measuring the blue fluorescence of the thiochrome produced on oxidation was applied by Westenbrink & Goudsmit [1937, 1] to the estimation of the vitamin in urine for clinical purposes. The specificity of the method seemed doubtful, as the figures they published in a later paper [Westenbrink & Goudsmit, 1938, 1] for the normal 24 hr. excretion were somewhat higher than those obtained by Harris *et al.* [1938] using the bradycardia method. Karrer [1937] and Ritsert [1938], using modified thiochrome methods, as well as many other workers more recently, reported values rather higher than those of Harris *et al.* [1938]. In an attempt to simplify the original method of Westenbrink & Goudsmit [1937, 1] for routine use, it was considered desirable to use the response to a standard test-dose, rather than the 24 hr. excretion, as an index of nutritional level. These authors have themselves examined the effect of test-doses [Westenbrink & Goudsmit, 1937, 2; 1938, 1, 2], mainly on the 24 hr. excretion, but suggest that the morning excretion, normally low after a light Dutch breakfast, may be used as a rapid test of nutritional level if 1 mg. of aneurin is given with the breakfast. Such a test has been made the basis of the present work, and comparison with the values obtained for the 24 hr. excretion show that the test can not only be completed more rapidly, but is also much more sensitive owing to the increased concentration in the urine. Wang & Harris [1939] have lately reported that the results they obtained by a modified thiochrome test agreed with those obtained on the same specimens of urine by biological assay.

Methods

(1) *Collection of specimens.* The urine was preserved at an acid pH, 0.5 ml. of conc. HCl or glacial acetic acid being added to the amount collected during each period of 3 hr. Toluene was added as well to prevent the growth of moulds. After collecting a 24 hr. specimen, a breakfast low in aneurin was given (e.g. white bread and butter, honey, tea), the urine being collected for a further 3 hr. A second 3 hr. specimen was then obtained after a similar meal supplemented with 1 mg. aneurin by mouth.

(2) *Analytical technique.* Normal urines and those from cases of suspected deficiency were filtered from deposits if necessary and diluted so that the amount excreted per hr. was made up to 500 ml. Specimens suspected of containing more than about 20 μ g. per hr. were diluted to contain less than this amount in 500 ml. The diluted specimens were brought to pH 3-5 if necessary. 75 ml. of the diluted urine were stirred mechanically in a centrifuge tube for 1-2 min. with 25 ± 2 mg. of "Clarit" acid clay (kindly supplied by the courtesy of Messrs Lever Bros.). A second aliquot was measured out and a similar amount of acid clay added during the stirring of the first. Two further tubes were set up to

prepare adsorbates from the same amount of urine with different amounts of added aneurin (less than $4\text{ }\mu\text{g.}$). The adsorbates were collected by centrifuging for 2–5 min. and the supernatant fluid was discarded. The wet adsorbates were treated in the same tubes with 2 ml. pure methyl alcohol. 1 ml. water was added to one of the tubes, which was to serve as a blank, prepared from urine without added aneurin. The contents of the four tubes were stirred with a stream of N_2 and 1 ml. $7.5\text{ }N$ NaOH was added to each, followed by 1 ml. 1.25% $\text{K}_3\text{Fe}(\text{CN})_6$ to each of the tubes except the blank. 12.5 ml. *isobutyl* alcohol saturated with water were added to each tube in turn and stirring was continued for 1–2 min. After thorough mixing the tubes were allowed to stand for a few minutes and the upper layer was decanted through a dry 8.5 cm. filter paper (previously purified by exhaustive extraction with wet *isobutyl* alcohol) into a test tube specially selected for fluorescence measurement and graduated for the collection of 10 ml. After the collection of 10 ml. of filtrate the tube was corked to prevent clouding through evaporation of some of the methyl alcohol during the fluorescence measurements. The fluorometer of Cohen [1935], constructed with improvements to be described later, was used. The intensity of the fluorometer lamp was standardized between readings by means of a solution of quinine sulphate in $0.1\text{ }N$ H_2SO_4 containing about 0.2 mg. quinine sulphate per 100 ml.

The aneurin content of the sample is most easily determined by a graphical method, illustrated in Fig. 1. Provided that the aneurin content is not too great, it is linearly related to the fluorescence (curve II) of the oxidized extracts. The intercept (ab) of this line on an abscissa at a level corresponding to the fluorescence of the unoxidized blank (abc) gives the aneurin content of the sample.

(3) *The fluorometer.* Only those details in which the instrument differs from that of Cohen [1935] are given. The source was a 500 watt low pressure Hg arc in a straight quartz tube and the beam was filtered through Wood's glass of 3 mm. thickness. The test tube was about 14 cm. from the source and as near as possible to the photoelectric cell. Heat from the source was absorbed by means of a fused quartz window in the lamp house instead of window glass. Scattered ultra-violet light was prevented from affecting the cell by means of a Wratten 2A filter. A suitable blue colour-filter was used to bring the maximum response of the cell to wave-length $460\text{--}470\text{ }\mu\mu$, corresponding to the maximum fluorescence of thiochrome [Kuhn & Vetter, 1935]. Using Weston "Photronic" cells, the makers' spectral sensitivity curves and the published transmissions of Wratten filters show that the type I cell requires a Wratten 49A filter while the type II cell requires a Wratten 48. The latter cell is more sensitive and has greater relative sensitivity in the blue, but is less permanent.

The test tubes used in the fluorometer were of thin non-fluorescent glass of nominal size $5 \times \frac{3}{4}\text{ in.}$ All tubes were rejected which did not conform to the following specifications:

- (a) External diameter less than 19 mm.
- (b) Internal diameter greater than 15 mm.

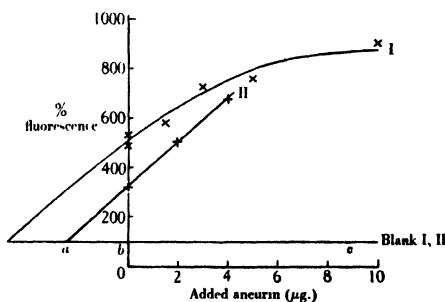


Fig. 1. Fluorescence and added aneurin. Case, H.F. iii. Specimen, 2. 3 hr. after 1 mg. aneurin. Curve I, 75 ml. dilution 0.50 l. per hr. Curve II, 75 ml. dilution 1.00 l. per hr.

(c) When graduated to contain 10 ml. the mark was at a height of 47.5 ± 1.0 mm.

(d) Fluorescence of 10 ml. quinine sulphate within $\pm 2\%$ of the mean. (More rigorous selection was impossible owing to fluctuation of the lamp intensity.)

(e) Constancy of the small fluorescence when they contained 10 ml. wet *iso*-butanol.

The galvanometer had an internal resistance of 189 ohms and a sensitivity of 3045 mm. per microamp. for 1 m. scale distance, but as this gave a deflexion of about 500 mm. per $\mu\text{g.}$ aneurin it was reduced to about 50 mm. per $\mu\text{g.}$ by means of a 1000 ohm shunt. These deflexions were obtained when using the more sensitive type II "Photronic" cell.

Discussion of the method

The technique adopted is closely similar to the simplification by Jowett [1939] of the original method of Westenbrink & Goudsmit. The methods agree in eliminating washing and drying of adsorbates, in the use of only one concentration of oxidizing agent and in the use of a blue-violet filter in the fluorescence measurement. Jowett, however, used a Zeiss-Pulfrich photometer for visual estimation of the fluorescence. Although this is an improvement on the simpler visual method first adopted by Karrer & Kubli [1937], we have rejected visual methods for routine use on account of errors due to the personal factor, in spite of the elimination of error due to fluctuations in the intensity of the source in the simple fluorometer of Cohen.

In order to give workers who have not used the thiochrome method some idea of the possible size of errors which may be involved, fairly detailed protocols are given in some of the tables illustrating various aspects of the method. The three types of specimen are denoted, for the sake of brevity as 24, 3_O and 3_{B₁} respectively. The fluorescence is expressed as the percentage of that produced under similar conditions from 1 $\mu\text{g.}$ aneurin oxidized in pure solution with 0.3 ml. 1.25% $\text{K}_3\text{Fe}(\text{CN})_6$.

(1) *Collection of specimens.* Oral administration of the test-dose was preferred, since after injection the very high concentration in the blood allows overflow through the kidney even in cases of deficiency [Westenbrink & Goudsmit, 1938, 1]. A 3 hr. test-period was chosen since it has been shown that the maximum excretion after a test-dose by mouth occurs during the 2nd hour. Collection for a 3rd hour was made to minimize errors due to the retention of urine formed previously but not voided, and the subject was encouraged to take enough fluid to ensure an output preferably not less than 100 ml. per hr. for the same reason. Although the test will not distinguish between inadequate reserves and faulty absorption, this is not of great clinical importance, since under the latter circumstances a conditioned deficiency will be present.

(2) *Adsorption.* Westenbrink & Goudsmit [1937, 1] found that the extra fluorescence from aneurin added to urine was often considerably less than that from aneurin oxidized in pure solution, although there was no significant loss due to incomplete adsorption. The fluorescence from added aneurin was increased by dilution, but under their conditions the apparent recoveries were only 57% for night urine diluted 10 times and 71% for day urine diluted 3 times. Using "Clarit" as adsorbent, with 24 hr. specimens diluted to 7.5 l. corresponding to the degrees of dilution used by Westenbrink & Goudsmit, the apparent recovery has varied from 33 to 74%. Increase of the dilution to 12 l. per 24 hr. gave the increased "recoveries" shown in Table I. Although the means for different

Table I. *Fluorescence from added aneurin oxidized in the presence of urine*

Nature of urine	No. of samples	Fluorescence	
		Mean per $\mu\text{g.}$	Standard deviation
N 24	6	87	14
N 3 _O	6	80	13
N 3 _{B₁}	6	81	25
H 24	8	69	18
H 3 _O	8	73	16
H 3 _{B₁}	8	74	10

N = normal. H = hospital case.

types of specimen show some uniformity, the variations between individual specimens, indicated by the standard deviation, emphasize the advisability of calibrating the fluorescence of added aneurin in the presence of each urine examined. Such calibration serves also to check the possibility of abnormal interference with the fluorescence which occurs in some specimens, and which might vitiate the value of the results for other reasons. Filtration of the specimens had been avoided, owing to the possibility of loss of aneurin by adsorption on the deposits or on the filter paper, until it was found that in the presence of deposits the fluorescence of the *isobutanol* extract, even in the case of the blank, was considerably reduced, presumably by non-fluorescent substances which reduced the intensity of ultra-violet light in the bulk of the solution by absorption. This effect is shown in Table II.

Table II. *Effect of filtration of urine on fluorescence*Subject H.F. iii. Sample 3_{B₁} (cf. Fig. 3, column 1)

	Fluorescence	
	Unfiltered urine	Filtered urine
Blank	58	108
Oxidized	69	169
Added aneurin (per $\mu\text{g.}$)	23	65
Apparent assay ($\mu\text{g.}$)	0.40	0.95

Table III. *Effect of amount of "Clarit" on fluorescence from added aneurin and on blank*

Subject	Nature of urine	Fluorescence of adsorbate			
		Blank with		Added vitamin B ₁ with (per $\mu\text{g.}$)	
		25 mg.	50 mg.	25 mg.	50 mg.
N.F. i	24	102	146	97	76
	3 _O	117	167	92	67
	3 _{B₁}	115	144	92	73
N.M. v	24	96	153	108	79
	3 _O	165	165	73	69
	3 _{B₁}	162	152	78	66

In order to reduce the adsorption of interfering substances as much as possible, not only is the maximum convenient dilution recommended, but the amount of adsorbent has also been reduced, as in the method of Marrack & Höllering [1939]. Table III shows that reduction of the amount of "Clarit" from 50 to 25 mg. per 75 ml. diluted urine not only increases the fluorescence per $\mu\text{g.}$ added aneurin but usually reduces the fluorescence of the blank. With

the smaller amount of adsorbent, the adsorption of aneurin is incomplete, since up to 25 % greater fluorescence is often obtained when aneurin is added to the adsorbate than when it is added to the corresponding amount of urine before adsorption (Table IV). Since the fluorescence increases linearly with the amount

Table IV. *Loss of aneurin on adsorption*

Subject	Nature of urine	No. of specimen	Fluorescence per $\mu\text{g.}$ vitamin B ₁ added to		% adsorbed
			Urine	Adsorbate	
N.M. i	24	2	80	83	96
H.M. iii	24	4	75	98	76
			72	96	
H.M. v	24	3	83	94	87
			82		
Mean =					86

of added aneurin, there must be a corresponding loss of the aneurin of the specimen for which compensation is made in the calibration, provided that this is carried out by adding aneurin to the urine before adsorption. If, for convenience in transmitting samples from a distance, adsorbates without added aneurin have been made, it has seemed advisable to increase the results by some 15–20 % to allow for the loss; this empirical correction must be regarded as an approximation only, since the loss seems to vary in different samples.

Wang & Harris [1939] have criticized the use of adsorption as a means of removing aneurin from interfering substances in urine and prefer to oxidize the urine directly. The method cannot be conveniently applied to aliquots of more than 2 ml. (i.e. 0.15 % of a 24 hr. specimen of average volume) and is therefore best adapted for use with concentrated urines of high aneurin content. For greater accuracy with urines of low aneurin content the present method which uses 0.625 % of a 24 hr. specimen as the normal aliquot is preferable. The method has been compared with that of Wang & Harris [1939] in the case of specimens of high aneurin content, and it has been found that with the same sample (2 ml.) the present method usually gives lower blanks and greater fluorescence per $\mu\text{g.}$ added aneurin, in spite of the fact that Wang & Harris [1939] claim quantitative recovery (Table V). This discrepancy is accounted for

Table V. *Comparison of the present method with that of Wang & Harris [1939]*

All determinations made on 24 hr. specimens. 2 ml. aliquot except in the case of H.M. v. 3 when 8.1 ml. were used for the determination by the present method

Specimen	Method	Fluorescence			Assay $\mu\text{g.}$	% of W. & H.
		Blank	Oxidized	1 $\mu\text{g.}$ vitamin B ₁		
*	Present	57	145	81	2.15	100
	W. & H.	68	145	71	2.15	100
*	Present	50	95	86	1.10	67
	W. & H.	51	110	71	1.65	100
H.M. ii. 3	Present	39	130	79	1.15	68
	W. & H.	68	178	64	1.70	100
H.M. v. 3	Present	34	224	83	2.30	57
		38	208	82	2.10	52
	W. & H.	36	100	63	1.00	100

* No reference no. given since the response to a test-dose was not determined and the case is not further discussed in this paper.

by the fact that these workers used a visual method by which it is impossible to assess the reduction of the fluorescence of thiochrome when added to the blank *isobutanol* extract of urine. Wang & Harris obtained the same results with their specimens as by the bradycardia method, while the present technique gives values on the average 30 % lower than those obtained by their thiochrome technique.

(3) *The blank test.* The use of an unoxidized sample as a blank is not entirely satisfactory, since the unknown substances responsible for this fluorescence might be either increased or reduced in amount during the oxidation of aneurin to thiochrome. The latter seems more likely, since it is frequently found that samples low in aneurin show diminished fluorescence on oxidation, unless a blue filter is used to eliminate, as far as possible, fluorescent light not due to thiochrome (Table VI). Ritsert [1938] avoided this difficulty by using as a blank

Table VI. *Effect of blue filter on assay*

Subject, H.M. iii (Fig. 3, col. 1, 24 hr. sample). Weston "Photronic" cell, type I

Wratten filters	2A	2A + 49A
Wave-length for maximum sensitivity	575 m μ	460 m μ
	Fluorescence	
Blank	159	77
Oxidized	149	98
Added aneurin (per μ g.)	73	65
Assay of aliquot (μ g.)	- 0.15	+ 0.30
Content of total (μ g.)	- 25	+ 50

that amount of an *isobutanol* extract prepared from urine freed from aneurin by adsorption which would give the same amount of white or greenish fluorescence as was judged by the eye to be present in the oxidized sample, which showed in addition the blue-violet fluorescence of thiochrome. Thiochrome was added to the blank till a match was obtained with the fluorescence of the *isobutanol* extract from an oxidized sample of the urine being tested. The amount of added thiochrome gave the aneurin content of the sample. The method is not theoretically sound since different urines differ qualitatively as well as quantitatively in the blank fluorescence. Moreover, the technique requires considerable visual acuity on the part of the observer and is likely to be profoundly susceptible to errors due to the personal factor. The average human eye, with sensitivity at 465 m μ less than 10 % of that at 555 m μ (the wave-length of maximum sensitivity), is less ideally adapted for the estimation of the fluorescence of thiochrome in the presence of a green blank than a selenium cell, even without a filter, for which the corresponding relative sensitivity may be 65-90 %, depending on the type of cell. Although the eye, unlike the photoelectric cell, distinguishes qualitatively between the green blank and the blue-violet of thiochrome, the presence of the two renders matching extremely difficult in any visual method (except that of Jowett [1939] using a violet filter), especially as the amount of green fluorescence may be different in the oxidized and unoxidized samples. Even with a filter the blank has a greater relative effect on the eye than on the selenium cell, owing to the smaller relative sensitivity of the former in the blue-violet region.

A further difficulty arising from the use of an unoxidized sample as a blank is due to the absorption of ultra-violet light by interfering substances, with a consequent reduction of the intensity of fluorescence in the bulk of the solution. If the concentration of such substances is altered during the process of oxidation,

the blank will differ from the oxidized samples in other respects than thiochrome content. Occasionally samples occur, especially with highly pigmented urines, in which visual observation detects practically no fluorescence in the layers of isobutanol extract remote from the source of light. As this interference is invariably reduced after oxidation, the oxidized sample shows an increase in fluorescence not due to the formation of thiochrome and the assay is consequently too high. A further type of interference which occurs in pigmented urines is due to traces of a yellow pigment which is present in the blank and absorbs some of the blue fluorescent light, but which is absent from or diminished in the extracts of the oxidized samples. An example is shown in Table VII in

Table VII. *Effect of pigment in blank extract*

Specimens, H.F. iii. i

Specimen	Fluorescence		
	24	*3 _O	3 _{B₁}
Blank	120	58	108
Oxidized	153	91	169
1 µg. added vitamin B ₁	83	60	65
Assay of aliquot (µg.)	0.40	0.55	0.95
Content of total (µg.)	65	11	19

* Blank extract orange, others pale yellow.

which the low blank for the 3 hr. specimen on a low aneurin intake (3_O) was due to pigment which was largely absent from the oxidized samples. As a result the apparent excretion is greater than the average for 3 hr. during the previous 24 hr. on a good hospital diet. It appears that the blank in this extreme case might be too low by 30 %.

Since it is impossible to estimate the effect of oxidation on the blank it is necessary to keep it as low as possible. In general this can be achieved by reducing the aliquot or by increasing the dilution when purification is carried out by adsorption. Accordingly, where the results obtained by the present method differ from those obtained by the method of Wang & Harris [1939], as shown in Table V, we believe them to represent more nearly the true aneurin content of the sample. Our lower blanks cannot be due to absorption of ultra-violet or fluorescent light, since such interference would give high results, while actually the results are lower than those obtained by the technique of Wang & Harris.

Reduction of the blank by reduction of the aliquot is limited by the minimum amount of aneurin which it is desired to detect. It has not yet been found possible to reduce the blank significantly by other means. After observing slightly lower blanks with samples freshly diluted at room temperature than with cold samples of the same dilution after a few hours in the ice-chest, the effect on the blank of adsorption at 37° was found to be only a very slight decrease.

Prolonged storage of samples may lead to a considerable increase in the fluorescence of the blank, its colour differing from that due to thiochrome (Table VIII, specimen N.M. ii. 24). The substances responsible are destroyed on oxidation since the fluorescence of oxidized samples is not increased and is the typical blue-violet of thiochrome, not the green of the blank. It is inadvisable to delay the preparation of adsorbates even though the aneurin itself may not be greatly reduced (specimen N.M. i. 3_{B₁}).

Table VIII. *Effect of storage*

The stored samples were diluted and kept at room temperature for 80–90 days

Specimen	Sample	Fluorescence	
		Fresh	Stored
N.M. ii. 24	Blank	89	131
	Oxidized	151	117
	1 μ g. vitamin B ₁	73	64
	Assay (μ g.)	0.85	–0.20
N.M. i. 3B ₁	Blank	86	71
	Oxidized	309	250
	1 μ g. vitamin B ₁	55	63
	Assay (μ g.)	4.05	2.85

(4) *Calibration.* For accurate calibration the amount of added aneurin must be at least equal to that present in the sample. On the other hand, if the total amount exceeds 7–10 μ g. (the actual value varying to some extent with different specimens) the linear relation between fluorescence and aneurin content fails as in curve I, Fig. 1, in which the sample was subsequently shown to contain 4.8 μ g. by the good linear curve with determinations at twice the normal dilution (Fig. 1, curve II). At the normal dilution the addition of an equivalent amount of aneurin shows an intensity of fluorescence already departing markedly from the linear relation.

The discrepancy between the fluorescence due to aneurin added to urine and that obtained when aneurin is oxidized in pure solution is an indication of the presence of interfering substances in the urine. Since, as shown above, the amount of such interference is not likely to have remained constant during the oxidation, the blank determination is subject to an uncertain degree of interference. Accordingly, where the apparent recovery of added aneurin is less than 60 % the result should be checked by determination with adsorbates made at a higher dilution, in which the ratio of interfering substance to aneurin is likely to have altered. Unfortunately, accurate comparison is only possible with values

Table IX. *Effect of dilution*

Subject, H.M. ii (Fig. 3, column 2). Aliquot, 75 ml. diluted urine

Specimen		Dilution, l. per hr.	
		0.50	1.00
		Fluorescence	
24	Blank	55	46
	Oxidized	163	108
	1 μ g. vitamin B ₁	42	74
	Assay of aliquot (μ g.)	2.60	0.85
	Content of total (μ g.)	415	270
3 ₀	Blank	44	47
	Oxidized	115	90
	1 μ g. vitamin B ₁	60	71
	Assay of aliquot (μ g.)	1.20	0.60
	Content of total (μ g.)	25	25
3 _{B₁}	Blank	45	39
	Oxidized	405	257
	1 μ g. vitamin B ₁	64	70
	Assay of aliquot (μ g.)	5.65	3.10
	Content of total	115	125

near the upper limit of the normal range, since increase of dilution reduces the maximum size of the aliquot which can be handled conveniently. An example is given in Table IX in which the two 3 hr. samples show good agreement at our standard and at twice our standard dilution. Since the low fluorescence of added aneurin in the 24 hr. sample was much improved by further dilution, the assay obtained in the latter case is much to be preferred.

(4) *The use of one concentration of oxidizing agent.* Since the amount of ferricyanide required for optimal increase of fluorescence depends on the total amount of oxidizable substances rather than on the amount of aneurin, attempts were made to avoid the need for determining the optimal conditions in each case by the use of excess, the redox potential being poised by the addition of ferrocyanide. No success was attained with ratios of oxidant to reductant between 0.7 and 40. It appeared that an initially high proportion of oxidant was required to oxidize the aneurin, but the fluorescence was reduced if such conditions were maintained by excess of a poised reagent.

In a series of determinations on 50 subjects requiring 0.1–0.4 ml. 5% $K_3Fe(CN)_6$, it was found that 31 cases required 0.2–0.3 ml., while for all cases, with and without added aneurin, the values with the latter amount were never lower than the optimal value by more than 10%. With significant amounts of aneurin in the sample, sub-optimal oxidation was compensated by similar sub-optimal oxidation of added aneurin in calibration. It was therefore decided to use 1 ml. 1.25% $K_3Fe(CN)_6$ which provides a slight excess in all cases.

Before increasing the sensitivity of the fluorometer to allow the use of a blue-violet colour filter, it was found that samples low in aneurin frequently gave an apparently negative assay under these conditions (e.g. Table VI). Such samples would have been reported as of zero content in any method based on maximum obtainable fluorescence. Since using the colour filter no such negative values have occurred, except after prolonged storage (Table VIII). Jowett [1939] also reported the harmless effect of excess of oxidizing agent when using a filter. It is evident that thiochrome is not significantly susceptible to excess of oxidizing agent under these conditions, as would be expected from the vast excess used in its preparation by Barger *et al.* [1935]. The error introduced by the oxidative destruction of substances responsible for the blank is considerably reduced by the use of a colour filter.

(5) *The use of wet isobutanol.* The solvent may be recovered in the wet state simply by steam-distillation after washing with water to prevent the accumulation of methyl alcohol which would alter the limits of miscibility with the alkaline aqueous phase. The wet solvent is advantageous in the removal of fluorescent substances from the filter-papers to be used in clearing the isobutanol extracts. Since its boiling-point is as low as 88° a continuous extractor heated on the water bath may be used. A form of extractor in which a column of papers is surrounded by the hot vapour is preferable to the Soxhlet extractor, recommended by Wang & Harris [1939], since extraction is more rapid and more complete.

Discussion of results

Fig. 2 summarizes the results obtained with a series of neurological cases of which the clinical diagnoses are given in Table X, and with a few normal controls who were research workers, except N.M. iv (technical assistant) and N.F. i (wife of N.M. v). The two series are arranged in ascending order of excretion after the test-dose. For ease of comparison with the excretion after the test-dose the 24 hr. excretion has been plotted as the average for 3 hr. Fig. 3 gives similar

data showing the increase of excretion for a number of patients undergoing treatment with aneurin, together with an example of the spontaneous variations which may occur in a normal case N.M. i.

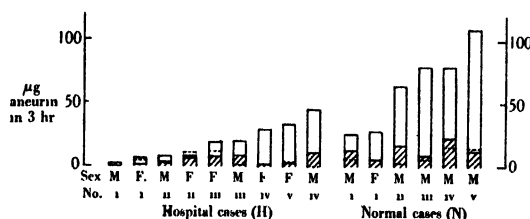


Fig. 2. Aneurin excretion and response to test-dose. Shaded rectangle = average 3 hr. excretion. Continuous rectangle = excretion after 1 mg. aneurin by mouth. A broken line indicates the level of excretion on a low aneurin intake.

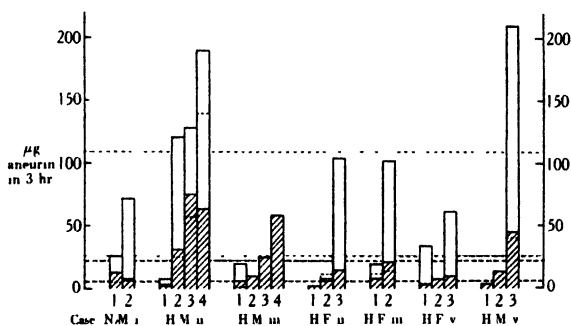


Fig. 3. Response of cases to treatment with aneurin. Details as in Fig. 2. - - - Normal limits for 3 hr. excretion after 1 mg. test-dose. --- Normal limits for average 3 hr. excretion during 24 hr. with dietary aneurin only.

Table X. *Clinical diagnoses of hospital cases*

Subject	Diagnosis
Males	
H.M. i	Polyneuritis
H.M. ii	Alcoholic periphe. neuritis
H.M. iii	Sub-acute combined degeneration with pernicious anaemia
H.M. iv	Polyneuritis
H.M. v	Peripheral neuritis
Females	
H.F. i	Nutritional polyneuritis
H.F. ii	Peripheral neuritis
H.F. iii	Polyneuritis
H.F. iv	Polyneuritis
H.F. v	Thyrotoxicosis and nutritional polyneuritis

The results for the 24 hr. excretion are roughly parallel with those obtained after the test-dose, but considerable variations occur, especially with urines of low aneurin content. It is impossible to say to what extent these variations are due to real differences in the aneurin content of the samples and how far they are affected by variations in the amounts of interfering substances. In an attempt to allow for this uncertainty determinations were made on the control samples excreted after a breakfast low in aneurin, in order to determine the actual response to the test-dose. This determination is, however, highly susceptible to errors due to incomplete emptying from the bladder of the con-

centrated urine of the previous night, which may contain much of the aneurin excretion of the previous 24 hr. [Westenbrink & Goudsmit, 1937, 2]. This is clearly the case with the specimens shown in Fig. 3, case H.M. ii, column 4, where the excretion on low aneurin intake is apparently more than twice as much as the average excretion during the previous 24 hr. Errors due to interfering substances cannot have affected these results significantly, since they were carried out at twice the normal dilution, the blank was low, namely, 5–12 % of the fluorescence of the oxidized samples and the fluorescence from added aneurin was high, 80–90 %. If retention of 70 $\mu\text{g.}$ out of a possible 150–200 $\mu\text{g.}$ in the night urine had occurred this would suffice to bring the “3₀” specimen to the level of the previous “24”. Anomalous values for other samples for the control period may also be due to this cause (Fig. 2, hospital cases, F. i, F. ii, F. iii). In all these cases, however, visual observation of the blank showed traces of yellow pigment and little fluorescence in the layers remote from the fluorometer lamp, though this interference was diminished or not detectable in the oxidized samples. The assays are evidently too high, as discussed already with special reference to case H.F. iii (Table VII). With case H.F. i, moreover, the value for the 24 hr. may be too low owing to the small volume (245 ml.). Similarly with H.M. i, the total absence of aneurin after the test-dose, in spite of its presence in the previous samples, may be due to the low volume of urine voided (60 ml.). Other anomalous figures, e.g. H.F. v and N.M. v, are not outside the limits of experimental error for the fluorometer measurement, which, for a specimen of low aneurin content determined under the standard conditions, might amount to $\pm 1 \mu\text{g.}$ calculated on a 3 hr. basis.

On account of these anomalies, the 3 hr. excretion after the test-dose, without correction for the excretion during the previous control period, is considered to be the best index of nutritional level when the thiochrome test is used. It is preferable that the test-period should be preceded by a few hours of low aneurin intake to avoid errors due to retention of the concentrated urine of the previous night.

It is not proposed to consider here the clinical significance of the results in detail as this will be discussed elsewhere (McAlpine, unpublished observations), but certain observations may be of value.

Owing to different metabolic needs and variations in kidney function, it is not to be expected that there will be a definite value above which deficiency can be said to be absent and below which clinical symptoms are apparent. This has already been pointed out by Harris *et al.* [1938]. Out of nine cases with neurological symptoms, Fig. 2 shows that the 24 hr. excretion of four was equal to or greater than the minimum for normal controls, i.e. 50 $\mu\text{g.}$ in 24 hr. or 6 $\mu\text{g.}$ in 3 hr. Only one of these (H.M. iv) showed a value after the test-dose which was higher than the minimum normal value observed here (26 $\mu\text{g.}$), but two other cases did so (H.F. iv and H.F. v). With regard to the 24 hr. values, apart from the possibility of greater relative influences of sources of error in the test, it must be borne in mind that the values are a function, not only of the reserves of the patient but also of the diet during the period of the test. Many of the cases may already be showing a response due to a good hospital diet even before treatment with supplements of aneurin. Thus H.F. ii (Fig. 3) assaying at 15 $\mu\text{g.}$ for the first 24 hr. (col. 1) gave an increased excretion of 50 $\mu\text{g.}$ in 24 hr. on the 5th day, although the excretion after a test-dose was still very small (col. 2). H.F. iv (Fig. 2), giving a dietary history indicating no gross deficiency and a fair response to the test-dose, showed a low 24 hr. excretion (15 $\mu\text{g.}$) due to a diet low in aneurin for the period of the test.

Although it appears that the thiochrome test may give rather uncertain information about the extent of hypovitaminosis, it can be of real value in the control of dosage and in the choice of the most economical route for effective administration. In the absence of such a test it has become customary to give 10 mg. or more per day, often parenterally. Westenbrink & Goudsmit [1938, 1] showed that more aneurin is lost through the kidney when it is given by injection than when given by mouth. The oral route is therefore preferable unless the possibility of faulty absorption is indicated by the absence of aneurin from the urine after a test-dose by mouth. Under such circumstances it is advisable to give aneurin parenterally until the deficiency is remedied sufficiently to give a moderate response to a test-dose orally administered. Further work on the absorption of aneurin is needed since it seems improbable that there should ever be difficulty in the absorption of such a small water-soluble molecule. In conditioned deficiencies due to incomplete utilization of the vitamin of the food, it seems possible that the difficulty may be in freeing it from protein with which it may be bound, as in milk [Houston *et al.* 1939], and possibly in animal tissues generally [Westenbrink & Goudsmit, 1938, 3].

Cases H.M. iii and H.F. iii (Fig. 3), admittedly showing only moderate deficiency when first examined, are examples of the response to oral treatment. H.F. iii received 1 mg. aneurin on the 2nd day as a test-dose and 3 mg. per day on the 5th, 6th and 7th days. 24 hr. specimens were collected on the 1st and 8th days and in each case were followed by examination of the response to a test-dose. The upper limit for the normal cases examined so far was reached by the criterion of either the "24" or the "3_B" specimen. H.M. iii received 1 mg. as test-dose on the 2nd day, 1 mg. per day on the 9th, 10th and 11th days and 3 mg. per day on the 12th, 13th and 14th days. The 24 hr. excretion was examined on the 1st, 8th, 11th and 14th days. By the 11th day (col. 3) it had reached the upper limit for normal cases and had reached the much higher figure of 465 μ g. by the 14th day. This is probably near the saturation level. H.M. ii and H.M. v reached similar levels after prolonged treatment, about 45 and 60 days respectively, receiving as much as 10 mg. per day by injection for much of these periods. The high levels of excretion, 500–600 and 350 μ g. per day respectively, are really due to reserves in the tissues and not directly due to excessively high aneurin intake, since none was given, apart from that in the good hospital diet, for 48 hr. before collecting the urine. Westenbrink & Goudsmit [1938, 1] give 400–500 μ g. as the saturation level for two normal subjects after treatment with 5 mg. per day for several days. The corresponding excretion after the test-dose was of the order of 200 μ g. in 3 hr., the response being less than 20 % of the dose. As this work was mainly concerned with establishing a rapid test for clinical purposes, the excretion subsequently has not been examined, but Westenbrink & Goudsmit showed that quantitative excretion is never attained, as after saturation with ascorbic acid [cf. Abbasy *et al.* 1936]. The fate of the aneurin not excreted intact is at present unknown; the capacity of the tissues for storing it is very limited and saturated subjects are rapidly depleted on a low aneurin diet.

Case H.F. v is an example of refractoriness to treatment, 10 mg. being given daily by intravenous injection for 6 days (4th–9th). The 24 hr. specimens were taken on the 1st, 3rd and 12th days. The failure to respond satisfactorily is probably due to the increased requirement for metabolism in thyrotoxicosis.

Since the normal values shown never reached the saturation level, it seems probable that the intake was sub-optimal although the individuals showed no symptoms. Other workers have reported normal ranges which reach higher

Table XI. *Normal daily excretion of aneurin*

Method	Range μg.	Mean μg.	Reference
Bradycardia	*35-105	*60	Harris <i>et al.</i> [1938]
Thiochrome	—	100	Karrer [1937]
Thiochrome	α120-330 β80-240	α230 β120	Westenbrink & Goudsmit [1938, 1]
Thiochrome	110-520	—	Ritsert [1938]
Bradycardia } Thiochrome }	*90-480	—	Wang & Harris [1939]
Thiochrome	50-170	100	This paper

* Reported as i.v. and converted by the relation, 1 i.v. = 3 μg.

α Male subjects.

β Female subjects.

levels (Table XI). While differences in the lower limits of the values obtained by the thiochrome test may be in part due to different amounts of interfering substances depending on the precise technique adopted, the upper values are less susceptible to such errors. Our values were determined in early spring and it is possible that reserves may have been depleted during the winter, owing to the greater demand for aneurin corresponding to greater calorie requirements, and to a diminished intake, due to possible deterioration in stored foods and to the lower content even of fresh foods, such as milk from stall-fed cattle as compared with pasture-milk (National Institute for Research in Dairying, *Annual Report*, 1938, p. 52).

In conclusion, it may be stated that the excretion of aneurin after a 1 mg. test-dose, determined by the thiochrome test, is a more reliable guide to the extent of hypovitaminosis-B₁ than the 24 hr. excretion, since it is proportionately less susceptible to errors arising from interfering substances in the urine. The patient who is considered to be suffering from a deficiency of aneurin may be given 1 mg. (i.e. 50-100 % of the normal daily requirement [Williams & Spies, 1938]) at the start of the test if a control period is omitted. The result can be available within 4 hr. of the start of the test; on the other hand, if the 24 hr. excretion is to be used as an index of nutritional level, the administration of aneurin must be postponed till the end of the period.

SUMMARY

1. A simplified procedure for the determination of aneurin in urine by the thiochrome test is described.
2. Photoelectric measurement of fluorescence is employed and rendered more specific for thiochrome by the use of a blue filter.
3. The importance of calibrating the fluorescence from aneurin in the presence of each specimen of urine examined is emphasized.
4. Six normal subjects excreted 50-170 μg. in 24 hr., the mean value being 100 μg. The excretion for 3 hr. after 1 mg. aneurin by mouth was 26-110 μg. with a mean of 65 μg.
5. Of 9 neurological cases, 5 gave a lower 24 hr. excretion and 6 gave a lower excretion after the test-dose than the lowest normal case.
6. The excretion of aneurin by individuals who were treated with aneurin, except for a case of thyrotoxicosis, increased considerably. Saturation appeared to be reached at a level of about 500 μg. per day. The corresponding excretion for 3 hr. after the 1 mg. test-dose was about 200 μg.

7. The excretion after a test-dose is suggested as the most reliable index of nutritional level, as its measurement is less susceptible to various sources of error which have been discussed.

I wish to thank Prof. E. C. Dodds, who suggested carrying out this work, and Dr D. McAlpine for the provision of neurological cases. The work was carried out during the tenure of a McKenzie-McKinnon Research Fellowship of the Royal Colleges of Physicians and Surgeons.

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CCXLV. PYRUVATE OXIDATION IN BRAIN

VII. SOME DIALYSABLE COMPONENTS OF THE PYRUVATE OXIDATION SYSTEM¹

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By the use of finely ground preparations (dispersions) of pigeon's brain in phosphate buffer, we have shown in a previous paper [Banga *et al.* 1939, 1] that cocarboxylase (vitamin B₁ pyrophosphate) is the active form of vitamin B₁ concerned with the oxidation of pyruvate in brain, and, further, that C₄ dicarboxylic acids activate this oxidation in a catalytic manner. The action of succinate on the oxidative decarboxylation of pyruvate by washed muscle preparations had been observed shortly before by Annau & Erdős [1939]. We shall show in this paper that, upon dialysing the brain dispersions, not only does the effect of the C₄ dicarboxylic acids become more definite, but the need for other substances in the oxidation system can also be demonstrated. This is the case with inorganic phosphate and "adenine nucleotide" (this term being here used to include adenylic acid and adenosine triphosphate). The necessity of "pyridine nucleotide" (cozymase) has been made likely. Further, it has been recently shown by Ochoa [1939] that magnesium (or manganese) ions are also indispensable components of the oxidation system. We now know that at least the following substances are components of the pyruvate oxidation system of brain and, probably, of other animal tissues: (1) cocarboxylase, (2) inorganic phosphate, (3) C₄ dicarboxylic acids (succinate, fumarate, etc.), (4) "adenine nucleotide", (5) Mg⁺⁺ (or Mn⁺⁺) and, probably, (6) cozymase (pyridine nucleotide).

It will further be shown that citrate or other intermediates of the citric acid cycle of Krebs & Johnson [1937], such as α -ketoglutarate, are much less active than C₄ dicarboxylic acids in the pyruvate oxidation system of brain, thus making it very unlikely that oxidation of pyruvate in brain can take place through such a cycle.

In another section the oxidation of phosphoglyceric acid by brain dispersions is studied. Phosphoglyceric acid is rapidly converted into pyruvic acid by the dispersions and is, therefore, readily oxidized. It has been found that the reactions

Phosphoglyceric acid \rightleftharpoons Phosphopyruvic acid,

Phosphopyruvic acid + Adenylic acid \rightarrow Pyruvic acid + Adenosine polyphosphate,

take place in brain in the same way as in muscle.

Experimental methods

Brain dispersions were prepared by thoroughly grinding, in an ice-cold mortar, 1 part of tissue with 4 parts of ice-cold 0.9% KCl; the mixture was then pressed through muslin. The dispersion was dialysed for various periods, under

¹ Preliminary report, Banga *et al.* [1939, 2].

constant mechanical shaking, in collodion tubes of narrow bore (taking about 10 ml. each) against 3 litres of 0.4% KCl, the temperature of which was kept between 1 and 3°. Unless otherwise stated the respiration experiments were carried out with 1.5 ml. samples of dialysed dispersion (equivalent to about 300 mg. brain) made up with additions (dissolved in water) to 2 ml. and buffered to pH 7.3. Both Barcroft and Warburg manometers have been used with air¹ in the gas space. Temperature 38° or 28°. Respiration experiments were mostly carried out in duplicate. The various additions were measured into the main space of the manometer bottles (best placed in ice) before the enzyme which was always added last. Readings were started after 5 min. temperature equilibration.

Pyruvate was determined colorimetrically by the salicylaldehyde method of Straub [1936]; the colorimetry was carried out against a freshly prepared standard of pure Na pyruvate using a Leitz compensation colorimeter.

Phosphate was determined colorimetrically by the method of Lohmann & Jendrassik [1926] and the pyrophosphate, phosphopyruvate and phosphoglycerate P fractions, by 7 min. hydrolysis at 100° in *N* HCl, hydrolysis in alkaline I₂, and difference between ashing and 180 min. hydrolysis in *N* HCl at 100° respectively.

Preparations

Freshly made up solutions of crystalline Na pyruvate were employed as usual.

Ba phosphoglycerate. Prepared from yeast by the method of Vercellone & Neuberg [1935]. Used as Na salt after decomposition with Na₂SO₄.

α-Ketoglutaric acid. A pure specimen prepared by Mr C. Long in this Laboratory.

Na oxaloacetate. A pure specimen from the Biochemical Laboratory of Szeged, Hungary.

Cozymase (90% pure). Prepared by Mr L. A. Stocken in this Laboratory by the method of Meyerhof & Ohlmeyer [1937].

Ba adenosinetriphosphate. A specimen from muscle kindly supplied by Prof. O. Meyerhof. Used as Na salt after decomposition with Na₂SO₄.

All other substances were commercial preparations including cocarboxylase (kindly presented by Messrs Merck and Co., U.S.A.) and adenylic acid (specimens from Messrs Fraenkel and Landau and Dr G. Henning).

All the acids were used as the Na salts.

We are much indebted to those mentioned for their help and presents.

1. EXPERIMENTS WITH PIGEON'S BRAIN

A. *Effect of phosphate, fumarate and "adenine nucleotide" on the oxidation of pyruvate*

After short periods of dialysis (2–3 hr.) the capacity of brain dispersions to oxidize added pyruvate is very small but their activity can be fully restored to the original value by addition of inorganic phosphate, fumarate and adenylic acid (or adenosinetriphosphate). To produce full reactivation the addition of all three substances is indispensable; if one of them is not added, pyruvate is no better oxidized than in the absence of all three. These facts are shown in Fig. 1, which gives in synthesis the results of many experiments of this type. Table I shows that adenylic acid can be replaced by adenosinetriphosphate.

¹ Pure oxygen was not better than air.

Table I. *Effect of adenosinetriphosphate (ATP) on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.01 M), fumarate (0.005 M), and pyruvate (0.0091 M). Air, 38°.

Time	μ l. O_2 uptake with		
	No addition	Adenylic acid (0.0014 M)	ATP (0.00035 M)
10 min.	55	186	186
20 min.	91	290	315

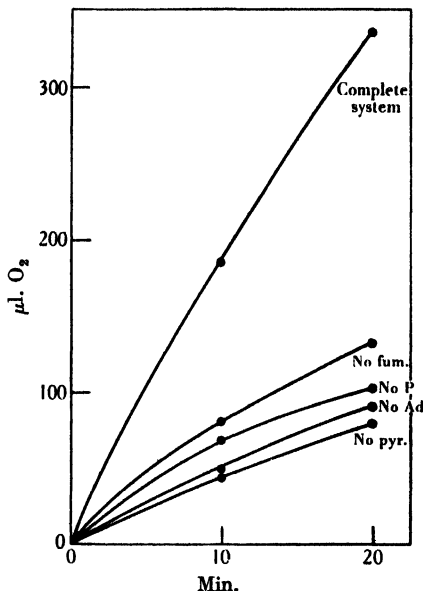


Fig. 1. Effect of phosphate (0.05 M), fumarate (0.005 M) and adenylic acid (0.00014 M) on the oxidation of pyruvate (0.0091 M) by dialysed dispersions from pigeon's brain. 1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$). The complete system contains enzyme + Mg^{++} + phosphate (P) + fumarate (fum.) + adenylic acid (Ad.) + pyruvate (pyr.). Sample with no phosphate buffered with $NaHCO_3$ to pH 7.3. Air, 38°.

If brains from B_1 -avitaminous pigeons (showing symptoms) are used, and the dispersions prepared from them dialysed as usual, cocarboxylase must also be added to obtain reactivation of the pyruvate oxidation system. In fact, in order to obtain marked activation by cocarboxylase, phosphate, fumarate and adenylic acid must all be added. The experiments of Table II show this for fumarate. The effect of cocarboxylase is small if only pyruvate, phosphate and adenylic acid are present but of the same order as before dialysis [cf. Banga *et al.* 1939, 1], if fumarate is also added.

That phosphate, fumarate and adenylic acid are necessary for the oxidation of pyruvate, and are not merely stimulating the oxidation of residual substrate which might be still present in the dispersions is clearly shown (1) by the fact (cf. Fig. 1) that the excess O_2 uptake of the complete system over the O_2 uptake of the samples containing everything but pyruvate is about three times larger than that of these samples, and (2) by the larger amounts of pyruvate disappearing when all the three substances are added to the dialysed dispersions (cf. Table III).

Table II. *Effect of cocarboxylase on the oxidation of pyruvate by dialysed dispersions from avitaminous pigeon's brain*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 M), and adenylic acid (0.00028 M). Air, 38°.

Exp.*	Cocarboxylase $\mu g.$	$\mu l.$ O_2 uptake in 20 min. with			
		No addition	Fumarate (0.005 M)	Pyruvate (0.0091 M)	Fumarate (0.005 M) + pyruvate (0.0091 M)
1	0.0	26	33	70	75
	0.5	57	74	93	215
Increase due to cocarboxylase		+31	+43	+23	+140
2	0.0	42	78	82	159
	0.7	45	84	120	261
Increase due to cocarboxylase		+3	+6	+38	+102

* 2 out of 4 experiments quoted.

 Table III. *Effect of phosphate, fumarate and adenylic acid on the aerobic disappearance of pyruvate in dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$) and pyruvate (0.0182 or 0.0091 M). Air, 38°.

Exp.	Phosphate (mol. $\times 10^{-3}$)	Fumarate (mol. $\times 10^{-3}$)	Adenylic acid (mol. $\times 10^{-3}$)	Pyruvic acid found after 20 min. mg.
1	—	5	0.14	3.65
	20	5	0.14	2.98
	50	5	0.14	2.85
	100	5	0.14	2.55
2	50	5	—	1.71
	50	—	0.14	1.42
	50	5	0.14	1.12
3	—	5	0.14	1.90
	50	—	0.14	1.50
	50	5	0.14	1.07

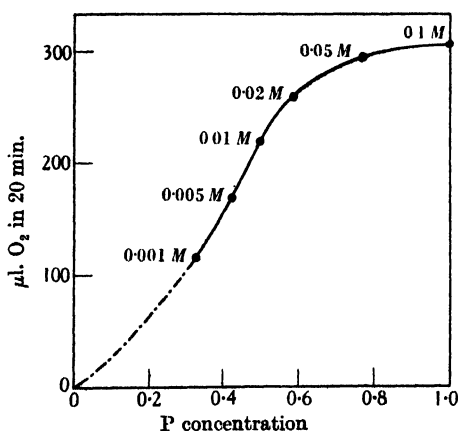


Fig. 2. Pyruvate oxidation in relation to the concentration of inorganic phosphate. O_2 uptake in 20 min. plotted against the reciprocal logarithms of the molar concentrations of phosphate. 1.5 ml. enzyme (pigeon's brain dispersion dialysed 2 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), fumarate (0.005 M), adenylic acid (0.00014 M), pyruvate (0.0091 M) and various concentrations of phosphate ($Na^+ - K^+$, pH 7.3). Air, 38°.

The effect of adenine nucleotide is obtained with catalytic concentrations. That the effect of fumarate and other C_4 dicarboxylic acids, although requiring rather larger concentrations to be optimum, is also a catalytic one, will be shown in the next section.

Optimum O_2 uptakes, on the other hand, require high concentrations of phosphate (0.05 to 0.1 M). The continuous line of Fig. 2 shows the relation between the O_2 uptake, in the presence of pyruvate, and phosphate concentration. After 2 hr. dialysis there is still an appreciable amount of phosphate present in the brain dispersions. The average value of several determinations corresponds to about 0.001 M phosphate (in 1.5 ml. dispersion diluted to 2 ml.) so that the point in Fig. 2 for 0.001 M phosphate corresponds to samples with no phosphate added (buffered with $NaHCO_3$ to pH 7.3). The extrapolation to zero (dotted line) is merely tentative, but it does not seem unreasonable to suppose that, at concentrations of phosphate much below 0.001 M , the O_2 uptake would be further significantly reduced.

B. Comparison of the catalytic actions of C_4 dicarboxylic, α -ketoglutaric and citric acids on pyruvate oxidation

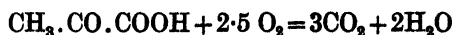
The net O_2 uptake of pyruvate in dialysed dispersions is always much greater in the presence of a C_4 dicarboxylic acid than in its absence; in fact, it is more than doubled in most cases (Table IV). The effects of succinate, fumarate and malate are practically identical. Smaller effects were obtained with oxaloacetate in one experiment, but this is probably due to the fact that part of it is decarboxylated to pyruvate with a consequent decrease in its optimum concentration.

The pronounced effect of the C_4 dicarboxylic acids is in marked contrast with the small activity of citrate. In Exp. 1 (Table IV), citrate had no effect whatever on the O_2 uptake of pyruvate, and in Exp. 2, 0.005 M citrate increased the net O_2 uptake of pyruvate by 36 μl . (from 110 to 146 μl .) or +33 %, whereas the same concentration of fumarate produced the almost four times greater rise of 131 μl . (from 110 to 241 μl .) or +120 %. In fact, citrate itself is not significantly oxidized by the brain dispersions.¹ As regards brei, this fact had already been observed by Long *et al.* [1939].

α -Ketoglutarate has also a smaller effect than the C_4 dicarboxylic acids on the oxidation of pyruvate by the dialysed brain dispersions (cf. Table IV, Exps. 4 and 7). Thus in Exp. 4, 0.005 M α -ketoglutarate increased the net O_2 uptake of pyruvate by 53 μl . (from 122 to 175 μl .) or +43 %, whereas the same concentration of fumarate increased it by 143 μl . (from 122 to 265 μl .) or +118 %. In Exp. 7 the effect of α -ketoglutarate is significantly greater than in Exp. 4, but also much smaller than that of fumarate. In the absence of pyruvate, α -ketoglutarate causes a significant increase of the O_2 uptake [cf. McGowan & Peters, 1937, for experiments with brain brei].

C. "Adenine nucleotide" and the molar oxygen/pyruvate ratio

The ratio of O_2 taken up to pyruvate disappearing was determined by McGowan [1937] using washed pigeon's brain brei. Whereas the theoretical value for complete oxidation of pyruvate according to the reaction



¹ In Exp. 1 (Table IV), 0.015 M citrate was added to the sample without pyruvate.

Table IV. *Comparison of the catalytic actions of C₄ dicarboxylic, α -ketoglutaric and citric acids on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂), phosphate (0.05 M), adenylic acid (0.00028-0.00014 M), and pyruvate (0.0091 M). Air, 38°.

Exp.	Duration of dialysis hr.	Sample	No addition	μ l. O ₂ uptake in 20 min. with						Citrate (0.005 <i>M</i>)
				Succinate (0.007 <i>M</i>)	Fumarate (0.005 <i>M</i>)	Malate (0.005 <i>M</i>)	Oxaloacetate (0.005 <i>M</i>)	α -Ketoglutarate (0.005 <i>M</i>)		
1	0.5	Enzyme	140	—	—	—	—	—	150*	—
		Enzyme + pyruvate	272	—	—	—	—	—	270	—
		Net O ₂ uptake of pyruvate	132	—	—	—	—	—	120 (0.0)	—
2	2.5	Enzyme	36	—	—	—	—	—	40	—
		Enzyme + pyruvate	146	—	—	—	—	—	186	—
		Net O ₂ uptake of pyruvate	110	—	241 (+ 120%)	—	—	—	146 (+ 33%)	—
3	2	Enzyme	34	—	—	81	80	—	—	—
		Enzyme + pyruvate	147	—	296	277	—	—	—	—
		Net O ₂ uptake of pyruvate	113	—	215 (+ 90%)	197 (+ 75%)	—	—	—	—
4	3	Enzyme	20	—	80	—	—	180	—	—
		Enzyme + pyruvate	142	—	345	—	355	—	—	—
		Net O ₂ uptake of pyruvate	122	—	265 (+ 118%)	—	175 (+ 43%)	—	—	—
5	3.5	Enzyme	25	67	—	—	—	—	—	—
		Enzyme + pyruvate	107	262	—	—	—	—	—	—
		Net O ₂ uptake of pyruvate	82	195 (+ 138%)	—	—	—	—	—	—
6†	3	Enzyme	27	57	59	—	—	—	—	—
		Enzyme + pyruvate	72	208	198	—	—	—	—	—
		Net O ₂ uptake of pyruvate	45	151 (+ 235%)	139 (+ 210%)	—	—	—	—	—
7‡	3	Enzyme	44	—	82	—	—	103§	—	—
		Enzyme + pyruvate	120	—	262	—	228§	—	—	—
		Net O ₂ uptake of pyruvate	76	—	180 (+ 137%)	—	125 (+ 65%)	—	—	—

* Citrate 0.015 M.

† Dispersion from B₁₂-avitaminous brain; all samples with cocarboxylase added.

‡ Dispersion from B₁₂-avitaminous brain; all samples with cocarboxylase added; also coenzyme (0.00004 M).

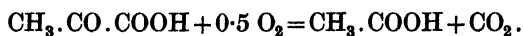
§ α -Ketoglutarate 0.0035 M. Figures in brackets give the percentage increase in the net O uptake of pyruvate due to the various acids compared.

is 2.5, McGowan found, as average of a number of experiments at 38°, 1.76, with extreme values of 1.60 and 1.98, indicating that the oxidation was not complete. Later Long [1938], also working with washed brain brei at 38°, found that production of acetic acid, and, to a much less extent, of lactic acid, practically accounted for the whole of the pyruvate not completely oxidized.

We have determined the O_2 /pyruvate ratio, in dialysed dispersions, in experiments at 38° and 28°, for respiration periods of 20 min. This short period was chosen because, at 38°, the rate of O_2 uptake in the dispersions (whether undialysed or dialysed), in the presence of pyruvate and all necessary additions, falls off very rapidly after 20–30 min., and becomes very small later. In fact, at 38°, the rate of O_2 uptake never remains constant over any reasonable length of time. Conditions are better at 28°; at this temperature, the rate of O_2 uptake, in the presence of pyruvate, remains constant for 40 min. and falls off markedly after this time (cf. Fig. 3). Some enzymic component of the oxidation system seems to be very unstable in the dispersions and is certainly highly sensitive towards higher temperatures.

The ratio mol. O_2 /mol. pyruvate in dialysed dispersions at 38°, has been found to be about 1.2, roughly half the theoretical value for complete oxidation, and approximately two-thirds of McGowan's value. At 28°, however, the ratio is about 1.5 which is much nearer to the values obtained with brain brei. The higher instability of some enzymic component of the system in the dispersions as compared with brei, referred to above, is probably responsible for the even less complete degree of oxidation of pyruvate in the former.

However, what is especially interesting here is the fact that the degree of oxidation of pyruvate by the dialysed dispersions is, in the absence of adenylic acid, much lower than in its presence. In fact, without adenylic acid, not only are both the O_2 uptake and the pyruvate disappearance much smaller but the ratio mol. O_2 /mol. pyruvate decreases to nearly 0.5, which is the theoretical value for the oxidative decarboxylation of pyruvate according to the reaction



This indicates that "adenine nucleotide" is also necessary for the oxidation of pyruvate beyond the stage of oxidative decarboxylation.

The experiments of Table V show that, under our experimental conditions, adenylic acid doubles the value of the O_2 /pyruvate ratio.

The two experiments at 38° of Table V have been carried out in duplicate, the experiment at 28°, with no adenylic acid added, in quadruplicate, and the

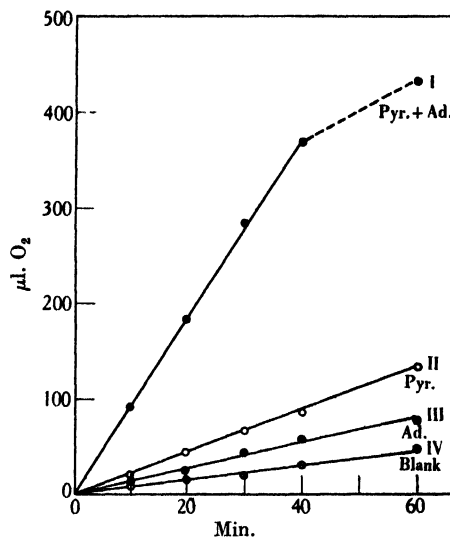


Fig. 3. Effect of adenylic acid on the oxidation of pyruvate by dialysed dispersions from pigeon's brain at 28°. 1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions. The complete system (curve I) contains enzyme, 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 M), fumarate (0.005 M), adenylic acid (0.00014 M) and pyruvate (0.0091 M). Curve II: no adenylic acid. Curve III: no pyruvate. Curve IV: no pyruvate, no adenylic acid. Air, 28°.

two experiments at 28°, with adenylic acid, in triplicate, both as regards measurements of O₂ uptake and pyruvate estimations. The results in the table are average values, the individual values varying not more than $\pm 5\%$.

The experiments were carried out by placing 0.2 ml. of 25 % trichloroacetic acid in the side bulbs of the Warburg bottles and stopping the reaction at the desired time by tipping in the trichloroacetic acid. For the zero time values of pyruvate¹ (both in experiments without or with adenylic acid) the trichloroacetic acid solution was tipped in after 5 min. temperature equilibration in the bath, immediately after taking the first reading of the other manometers. In the experiment at 28°, without adenylic acid, as the pyruvate disappearance in 20 min. would be very small and, therefore, difficult to estimate accurately, the respiration was continued for 1 hr. and the final pyruvate value determined at the end of this period. During this time the O₂ uptake increases linearly with time (cf. Fig. 3, curve II), in other words, the respiration rate is constant. The rate of pyruvate disappearance must have also been constant and the ratio O₂/pyruvate after 20 min. must have been the same as after 60 min. The values for the pyruvate disappearance in 20 min. given in Table V, have been calculated on this assumption.

Table V. *Effect of adenylic acid on the O₂/pyruvate ratio in dialysed dispersions from pigeon's brain at 38° and 28°*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂), phosphate (0.05 M), fumarate (0.005 M), and pyruvate (0.0091 M). Air.

Temp.	Adenylic acid (mol. $\times 10^{-3}$)	Extra O ₂ uptake due to pyruvic acid in 20 min.		Pyruvic acid disappeared in 20 min.		mol. O ₂ mol. pyruvic acid
		μ l.	μ mol.	mg.	μ mol.	
38°	—	43	1.920	0.342	3.880	0.495
	0.14	215	9.580	0.698	7.930	1.21
38°	—	47	2.100	0.270	3.070	0.68
	0.14	254	11.400	0.910	10.410	1.10
28°	—	15	0.656	0.077	0.873	0.75
	0.14	157	7.000	0.436	4.950	1.42
	0.14	131	5.850	0.348	3.960	1.48

After removing the protein precipitate by centrifuging the pyruvate was determined on 1 ml. aliquots of the clear filtrate, as described in the section on experimental methods.

The net O₂ uptake of pyruvate was always determined as the difference between control samples and those with added pyruvate, either with or without adenylic acid.

Table VI. *O₂/pyruvate ratio of the extra metabolism produced by adenylic acid in the experiments at 28° of Table IV*

Extra O ₂ uptake caused by adenylic acid in 20 min.		Extra pyruvic acid disappearance caused by adenylic acid in 20 min.		mol. O ₂ mol. pyruvic acid
μ l.	μ mol.	mg.	μ mol.	
177	7.890	0.349	3.970	1.99
139	6.200	0.246	2.790	2.22

In the experiments at 28°, the extra O₂ uptake (with pyruvate) and the extra disappearance of pyruvate caused by adenylic acid were also determined.

¹ Always duplicates at least

In this way the O_2 /pyruvate ratio of the excess metabolism produced by adenylic acid can be estimated. As shown in Table VI this ratio is considerably nearer the theoretical value of 2.5 for complete oxidation. This means that the extra pyruvate disappearing under the action of adenylic acid is being much more completely oxidized to CO_2 and H_2O .

D. Effect of cozymase

As already reported [Banga *et al.* 1939, 2] addition of cozymase to samples containing 0.00014 *M* adenylic acid has little or no effect when the enzyme has been dialysed for periods of 2–3 hr., but causes significant increases of the O_2 uptake after longer periods of dialysis. Since then it has been found that, on the one hand, cozymase (90 % purity) can completely replace adenylic acid. This is the case (with pigeon's brain dispersions) even after 15 hr. dialysis (Table VII).

Table VII. *Effect of cozymase on the oxidation of pyruvate by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 *M*), fumarate (0.005 *M*), and pyruvate (0.0091 *M*). Air, 38°.

Exp.	Duration of dialysis hr.	Adenylic acid (mol. $\times 10^{-3}$)	Cozymase (mol. $\times 10^{-3}$)	μ l. O_2 uptake in 20 min.
1	8	—	—	71
		0.14	—	130
		2.80	—	131
		0.14	0.14	190
2	3	—	—	95
		0.14	—	235
		0.28	—	243
		—	0.14	343
		—	0.28	390
3	7.5	—	—	61
		1.4	—	250
		—	0.28	270
		1.4	0.28	287
4	6	0.14	—	136
		1.4	—	230
		—	0.14	180
		1.4	0.14	240
5	15	—	—	57
		0.07	—	84
		0.14	—	103
		0.07	0.035	118
		0.28	—	172
		—	0.28	203
		0.14	0.07	180
		0.14	0.14	187
		0.70	—	188
		0.14	0.21	198

On the whole cozymase is, molecule for molecule, more active than adenylic acid (Table VII, Exps. 2, 4, 5). On the other hand, after periods of dialysis of 6 hr. or over, it frequently happens that increase of the adenylic acid beyond 0.00014 *M* produces a marked increase of the O_2 uptake (Table VII, Exp. 4), when further addition of cozymase has little, if any, effect. Occasionally, however, the higher concentrations of adenylic acid do not give rise to increased respiration which can then be obtained with cozymase (Table VII, Exp. 1).

In view of these fresh facts the question as to whether cozymase is indispensable in the pyruvate oxidation system of brain obviously cannot be solved

unless the brain dispersions could be submitted to much longer periods of dialysis without irreversible inactivation of the enzyme proteins. Recently we have been able to dialyse the brain dispersions for periods over 15 hr., up to 24 hr., after which time the activity of the enzyme, after addition of Mg^{++} , phosphate, fumarate and adenylic acid, is only about 25% of the original. Even then (with 0.0007 *M* adenylic acid) cozymase produced little effect. In this connexion we might recall that Meyerhof & Ohlmeyer [1936; 1937], using acetone powder extracts from muscle, obtained full reactivation of the lactic acid formation, in the partial reaction triosephosphate + pyruvate, after 15 hr. dialysis, by adding Mg^{++} and adenylic acid. Only after 36–48 hr. dialysis was addition of cozymase indispensable and, even so, less cozymase was required to produce a certain degree of reactivation when adenylic acid was present. They point out that, in order to prove the indispensability of cozymase in their system, the cozymase had to be reduced to under 1/1000 of the amount originally present in the enzyme preparations. When this was attained, however, the capacity of the extracts to form lactic acid from glycogen was irreversibly lost.

On the whole our experiments seem to lend some support to the view that cozymase is another component of the pyruvate oxidation system of brain. In addition, if the reaction oxaloacetate \rightleftharpoons malate is playing a catalytic part here, as it does in the oxidation of triosephosphate in muscle [Szent-Györgyi, 1937], the necessity of cozymase is evident.

Another question is how can cozymase replace adenine nucleotide. Either it can function as cophosphorylase [cf. Ohlmeyer & Ochoa, 1937] or it is broken down to some extent by the brain dispersions with formation of cophosphorylase ("adenine nucleotide"). Experiments on the liberation of inorganic phosphate from phosphoglyceric acid, to be reported in the next section, would seem to favour the latter view.

E. Oxidation of phosphoglycerate

If phosphoglyceric acid is used as substrate, the O_2 uptake with dialysed brain dispersions, after addition of Mg^{++} , phosphate, fumarate and adenylic acid, is not much lower than with pyruvic acid (Table VIII). In the two experiments of Table VIII (out of several similar experiments), the average net O_2

Table VIII. *Oxidation of phosphoglyceric acid by dialysed dispersions from pigeon's brain*

1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 *M*), fumarate (0.005 *M*) and adenylic acid (0.00028 *M*). Air, 38°.

Exp.	NaF <i>M</i>	Pyruvate <i>M</i>	Phosphoglycerate <i>M</i>	$\mu l.$ O_2 uptake in 20 min.
1	—	—	—	70
	0.04	—	—	43
	—	0.0091	—	348
	0.04	0.0091	—	242
	—	—	0.018	257
2	0.04	—	0.018	40
	—	—	—	65
	—	0.0091	—	310
	—	—	0.018	270

uptake of pyruvate in 20 min. was 260 $\mu l.$, and that of phosphoglycerate 196 $\mu l.$, or 75% of the former. The observation of Jowett & Quastel [1937], that phosphoglycerate increased but slightly the O_2 uptake of guinea-pig brain slices, must

have been due to poor penetration of the compound. Similar negative results (from 1935 onwards) have repeatedly been obtained in this laboratory with pigeon's brain brei using either phosphoglyceric or phosphopyruvic acid. This recalls the failure to obtain cataturulin effects with small amounts of cocarboxylase in brain brei or slices, whereas they are easily obtained in dispersions [cf. Banga *et al.* 1939, 1].

Phosphoglyceric acid is not directly oxidized. If its conversion into phosphopyruvic acid is prevented by NaF, it produces no increase whatever of the O_2 uptake (Table VIII, Exp. 1); under identical conditions the oxidation of pyruvate is only inhibited by 40% [cf. Peters *et al.* 1935]. In fact, the oxidation of phosphoglycerate is due to its rapid conversion into pyruvate¹ and inorganic phosphate.² This conversion had been observed to take place with rabbit's brain extracts by Mazza & Valeri [1935]. The additional points in Table IX are the

Table IX. *Liberation of inorganic phosphate from phosphoglyceric acid in dialysed dispersions from pigeon's brain*

1 ml. enzyme (dialysed 6 hr.) to 1.5 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.0066 *M*), and phosphoglycerate (0.017 *M*). Air, 38°. Ad = adenylic acid. Coz = cozymase. Pgl = phosphoglyceric acid. Ppyr = phosphopyruvic acid.

Time min.	Ad added (mol. $\times 10^{-3}$)	Coz added (mol. $\times 10^{-3}$)	Inorganic P mg.	Pgl P mg.	Ppyr P mg.	Increase of inorganic P mg.
0	—	—	0.37	0.74	0.00	—
30	—	—	0.40	0.51	0.20	0.03
30	0.14	—	1.16	0.00	0.00	0.79
30	—	0.28	1.18	0.00	0.00	0.81

formation of iodine-labile P (phosphopyruvate-P) in the absence of "adenine nucleotide", and the obvious necessity of this (in catalytic concentrations) for the reaction causing liberation of inorganic phosphate. The occurrence of this reaction and the high rate at which it takes place³ indicates that the brain dispersions contain a strongly active adenosinetriphosphatase. This makes it impossible to decide whether oxidation of pyruvate in brain dispersions is accompanied by phosphorylation of adenylic acid to adenosinepolyposphate. When dialysed brain dispersions are incubated with adenylic acid containing

Table X. *Transfer of phosphate from phosphoglyceric acid to adenylic acid in dialysed extract from pigeon's brain*

1 ml. enzyme (dialysed 3 hr.) to 1.5 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.0066 *M*), phosphoglycerate (0.017 *M*; 0.8 mg. P), and adenylic acid (0.0028 *M*; 0.35 mg. P). Air, 30°.

Time min.	Inorganic P mg.	Phosphopyruvic P mg.	Pyrophosphate P mg.
0	0.35	0.00	0.00
15	0.44	0.03	0.39

0.17 mg. P, at 30° for 5 min., some easily hydrolysable P (7 min., 100°, *N* HCl) accumulates in the presence, but not in the absence, of pyruvate; the quantities

¹ Detected qualitatively by the nitroprusside reaction.

² With preparations of *Bacterium Delbrückii*, which oxidize pyruvic acid to acetic acid and CO_2 , Lipmann [1937] found that phosphopyruvic acid was not oxidized.

³ Liberation of PO_4 was found to be complete in 10 min. in other experiments.

formed are, however, very small, generally amounting to only about 0.01 mg. P. On the other hand, the adenosinetriphosphatase is insoluble¹ and, when the dispersions are centrifuged at high speed (15,000 rev./min.) for a short time, the clear solution is no longer able to set free inorganic phosphate from phosphoglyceric acid significantly; but as the enzymes catalysing the conversion of phosphoglyceric into phosphopyruvic acid and transferring phosphate from the latter to adenylic acid are present in it, adenylic acid is readily phosphorylated (Table X).

With the demonstration of the formation of phosphopyruvic from phosphoglyceric acid, and the transfer of phosphate from phosphoglyceric to adenylic acid in brain preparations, which completes the evidence supplied by the experiments of Mazza & Valeri [1935], Euler *et al.* [1936] and Meyerhof [1938], we now have the final proof for the existence in brain of a glycolytic enzyme system which, at least qualitatively, is identical with that of skeletal muscle. Evidence in favour of a similar glycolytic mechanism in the retina has been recently advanced by Süllmann & Vos [1939].

As shown in Table IX, cozymase is as active as adenylic acid in catalysing the liberation of inorganic phosphate from phosphoglyceric acid in brain dispersions. This is not the case with muscle extracts [Schlenk *et al.* 1937], hence it seems likely that brain dispersions split cozymase to some extent with liberation of cophosphorylase.

2. EXPERIMENTS ON MAMMALIAN BRAIN AND KIDNEY (with technical assistance by R. W. Wakelin)

Substantially the same results as with pigeon's brain dispersions, have been obtained with dispersions prepared from rabbit's brain. The oxidation of pyruvate, after dialysis, requires the addition of phosphate, fumarate and adenine nucleotide as shown in Table XI. The difference from pigeon's brain dispersions is that, whereas, after periods of dialysis not exceeding 1.5 hr., cozymase is practically as active as adenylic acid, and can replace it molecule for molecule (Table XI, Exp. 2a), its activity is enormously reduced after dialysis periods of over 4 hr. (Table XI, Exps. 2b, 3b). Thus it seems that, in rabbit's brain dispersions, the enzyme splitting off cophosphorylase from cozymase is easily inactivated by dialysis; in general the rabbit preparations seem less stable after dialysis.

The effect of fumarate and adenylic acid has also been obtained in dispersions from guinea-pig's brain, even without dialysis² (Table XII), but we have failed to obtain active dispersions from rat's brain even with several modifications. Although the O₂ uptake of both brei and slices of rat brain is markedly increased by pyruvate, this is only so to a very small extent with dispersions. The explanation for this must probably be sought in the much higher instability of some enzymic component of the system.

In preliminary experiments with dispersions of rabbit kidney cortex, the necessity of fumarate and adenylic acid in the pyruvate oxidation system has also been demonstrated. The effect of adenylic acid is shown in Fig. 4. Table XIII shows the large increase in the net oxidation of pyruvate obtained when both

¹ The same finding, as regards the adenosinetriphosphatase of muscle, has been recently reported by Engelhardt & Ljubimowa [1939].

² In two experiments out of three.

fumarate and adenylic acid are added. The kidney dispersions were prepared in exactly the same way as those of brain, except that 1 part tissue was ground with only 2 volumes of 0.9% KCl.

Table XI. *Effect of phosphate, fumarate, adenylic acid and cozymase on the oxidation of pyruvate by dialysed dispersions from rabbit's brain*

1.5 ml. enzyme* (dialysed for various periods) to 2 ml. with additions including 0.1 mg. Mg⁺⁺ (as MgCl₂). Air, 38°. (5 out of 10 experiments quoted.)

Exp.	Duration of dialysis hr.	(mol. $\times 10^{-3}$)					μ l. O ₂ uptake in 20 min.
		Pyruvate	Phosphate	Fumarate	Adenylic acid	Cozymase	
1	3.75	—	50	5	0.14	—	27
		9.1	—	—	0.14	—	37
		9.1	50	—	0.14	—	52
		9.1	—	5	0.14	—	54
		9.1	50	5	0.14	—	153
2a†	1.5	—	50	5	0.14	0.14	59
		9.1	50	5	—	—	58
		9.1	50	5	0.14	—	198
		9.1	50	5	—	0.14	153
		9.1	50	5	0.14	0.14	224
2b	4.5	—	50	5	0.14	0.14	30
		9.1	50	5	—	—	46
		9.1	50	5	0.14	—	163
		9.1	50	5	—	0.14	62
		9.1	50	5	0.14	0.14	204
3a‡	1.25	—	50	5	1.4	0.14	59
		9.1	50	5	—	—	48
		9.1	50	5	1.4	—	254
		9.1	50	5	—	0.14	140
		9.1	50	5	1.4	0.14	244
3b‡	4.5	—	50	5	1.4	0.14	42
		9.1	50	5	—	—	53
		9.1	50	5	1.4	—	175
		9.1	50	5	—	0.14	65
		9.1	50	5	1.4	0.14	185
4§	4	—	50	5	0.14	—	30
		9.1	50	—	—	—	35
		9.1	50	5	—	—	46
		9.1	50	—	0.14	—	41
		9.1	50	5	0.14	—	99
5a	0.7	—	50	5	0.7	0.14	94
		9.1	50	5	—	—	62
		9.1	50	5	0.7	—	271
		9.1	50	5	—	0.14	183
		9.1	50	5	0.7	0.14	270
5b	1.7	—	50	5	0.7	0.14	63
		9.1	50	5	—	—	64
		9.1	50	5	0.7	—	249
		9.1	50	5	—	0.14	164
		9.1	50	5	0.7	0.14	248

* The amount of brain tissue equivalent to 1.5 ml. enzyme varied in the different experiments (from 190 to 316 mg.) but the O₂ uptake has been referred throughout to 300 mg. tissue for better comparison with the experiments on pigeon's brain.

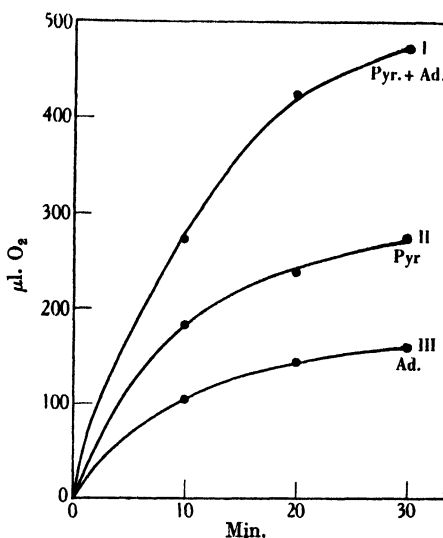
† 2a and 2b, 3a and 3b, and 5a and 5b represent respectively samples from the same brain. In another experiment the cophosphorylase effect of cozymase had practically disappeared after 1.75 hr. dialysis.

‡ Dialysis against 0.9% KCl.

§ Malate used instead of fumarate.

Table XII. *Effect of fumarate and adenylic acid on the oxidation of pyruvate by undialysed dispersions from guinea-pig's brain*1.5 ml. enzyme to 2 ml. with additions including phosphate (0.05 *M*). Air, 38°.

Pyruvate (mol. $\times 10^{-3}$)	Fumarate (mol. $\times 10^{-3}$)	Adenylic acid (mol. $\times 10^{-3}$)	μ l. O ₂ uptake in 20 min.
—	0.5	0.14	71
9.1	—	—	61
9.1	0.5	—	82
9.1	0.5	0.14	197

Fig. 4. Effect of adenylic acid on the oxidation of pyruvate by dialysed dispersions from kidney cortex (rabbit). 1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions. The complete system (curve I) contains enzyme, 0.1 mg. Mg^{++} (as $MgCl_2$), phosphate (0.05 *M*), fumarate (0.005 *M*), adenylic acid (0.0028 *M*) and pyruvate (0.0091 *M*). Curve II: no adenylic acid. Curve III: no pyruvate. Air, 38°.Table XIII. *Effect of fumarate and adenylic acid on the oxidation of pyruvate by dialysed dispersions from kidney cortex (rabbit)*1.5 ml. enzyme (dialysed 3 hr.) to 2 ml. with additions including 0.1 mg. Mg^{++} (as $MgCl_2$), and phosphate (0.05 *M*). Air, 38°.

Sample	μ l. O ₂ uptake in 20 min.	
	No addition	Fumarate (0.005 <i>M</i>) + Adenylic acid (0.0028 <i>M</i>)
Enzyme	42	160
Enzyme + pyruvate (0.0091 <i>M</i>)	172	472
Net O ₂ uptake of pyruvate	130	312

Increase of O₂ uptake of pyruvate in presence of fumarate + adenylic acid 182 μ l. (+ 140%).

DISCUSSION

The role of inorganic phosphate and "adenine nucleotide" in pyruvate oxidation.
 The necessity of both inorganic phosphate and adenine nucleotide for the oxidation of pyruvate in animal tissues, which is here shown for the first time, leads to the hitherto unsuspected assumption that phosphorylation reactions must play a part in oxidative breakdown of carbohydrate, beyond the stage of

phosphopyruvic acid. As regards bacteria Lipmann [1937] had shown that inorganic phosphate is required for the oxidative decarboxylation of pyruvate to acetic acid and CO_2 by washed acetone preparations of *Bacterium Delbrückii*; the possibility that a phosphorylation to phosphopyruvic acid, prior to the dehydrogenation, might be necessary was excluded by his finding that phosphopyruvic acid itself was not oxidized by the bacterial preparations. Lipmann [1938] observed later that decarboxylation of pyruvic acid by yeast does not require inorganic phosphate. More recently [Lipmann, 1939, 1] he was able to show that, if added to the (adenosinetriphosphatase-free) bacterial preparations, in the presence of pyruvate, adenylic acid is phosphorylated to adenosinepolyphosphate.

Lipmann's observations, however, differ from our own in one important respect. In the absence of adenylic acid, the dehydrogenation of pyruvate by the bacterial enzymes was not lower than in its presence, as one would expect if there existed a true coupling between pyruvate dehydrogenation and the phosphorylation of adenylic acid [cf. for instance, Meyerhof, 1937]. In our experiments with brain dispersions, on the other hand, the oxidative removal of pyruvate is very small in the absence of adenylic acid and is greatly increased when the nucleotide is added. Furthermore, the net O_2 uptake of pyruvate is increased to an even greater extent, so that pyruvate is being more completely oxidized. Thus, whereas "adenine nucleotide" may be necessary for the oxidative decarboxylation of pyruvate in animal tissues, it also seems to be required in further oxidative stages or other reactions involved in the oxidation.

The possibility that, in muscle respiration, phosphorylation may be coupled with reactions other than the dehydrogenation of triosephosphate [cf. Lennerstrand, 1937; Needham & Lu, 1938], is suggested by experiments of Belitzer [1939], who found that addition of creatine to muscle brei suspensions in phosphate led to both increased O_2 uptake and synthesis of phosphocreatine. This happened with lactate as substrate, although to a less extent, even in the presence of 0.00025 *M* bromoacetate which completely inhibited glycolysis. Whether such low concentrations of bromoacetate would also inhibit aerobic oxidation of triosephosphate is not stated.

If pyruvate oxidation is coupled with phosphorylation of "adenine nucleotide", then the energy of the main reaction of respiration would also seem to be made available through the adenylic acid and creatine phosphate systems.

Quite recently Lipmann [1939, 2] has suggested acetylphosphate as an intermediate in the dehydrogenation of pyruvate by *B. Delbrückii* preparations and, possibly, by animal tissues, but Ochoa *et al.* [1939] could not find evidence to support the latter view. The evidence at present available favours the possibility of an intermediary phosphorylation in the oxidation of pyruvate by both bacteria and animal tissues and, in spite of differences in the experimental results, it would seem that a common phosphorylated intermediate, which does not appear to be acetylphosphate, might be formed in both cases [Ochoa *et al.* 1939]. Recent experiments by one of us¹ show that, in brain dispersions poisoned with NaF, and in presence of pyruvate and the other components of the oxidation system, there is some disappearance of inorganic phosphate with equivalent formation of an easily hydrolysable ester, which is neither alkali- nor iodine-labile. The absolute amounts of ester formed are small but the ratio mol. pyruvate disappeared/atoms P esterified is only about 3. Further experiments in this direction are in progress.

¹ Ochoa, unpublished results.

The role of C₄ dicarboxylic acids. Whether the system of C₄ dicarboxylic acids is only acting in a first stage of pyruvate dehydrogenation or whether (as is the case with "adenine nucleotide") it is also involved in further stages, has not been accurately investigated, but we have some evidence that the latter is the case.

On the other hand, it appears that C₄ dicarboxylic acids are acting, in brain pyruvate oxidation, in the way suggested by Szent-Györgyi and his collaborators [cf. Szent-Györgyi, 1937], i.e. in a reversible catalytic system of hydrogen carriers, rather than as intermediates in a catalytic cycle such as the citric acid cycle of Krebs & Johnson [1937]. If the latter occurred one would expect citric acid (also α -ketoglutaric acid) to be at least as active as any of the C₄ dicarboxylic acids. This is not the case in spite of the fact that brain contains both aconitase [Johnson, 1939] and isocitric dehydrogenase [Adler *et al.* 1939].

SUMMARY

1. Dialysis of pigeon brain dispersions for short periods greatly reduces their power to oxidize pyruvate. Full reactivation can, however, be obtained by addition of inorganic phosphate + a C₄ dicarboxylic acid (succinate, fumarate, malate) + "adenine nucleotide" (adenylic acid or adenosinetriphosphate). The same is true for dispersions of mammalian brain (rabbit).

2. In the presence of the other components of the system, adenine nucleotide not only increases the oxidative removal of pyruvate considerably, but makes its oxidation more complete. Thus, whereas in the absence of adenine nucleotide the ratio mol. O₂ taken up/mol. pyruvate removed is 0.75, in its presence, the ratio is increased to 1.5 (at 28°).

3. The ratio mol. O₂/mol. pyruvate of the excess metabolism produced by adenylic acid is 2.1, i.e. near the theoretical value of 2.5 for complete oxidation of pyruvate to CO₂ + H₂O.

4. Cozymase can replace adenylic acid and (in pigeon's brain dispersions) the former is, molecule for molecule, even more active than the latter. This effect of cozymase is probably due to enzymic splitting of cophosphorylase ("adenine nucleotide"). In rabbit's brain dispersions, cozymase replaces adenylic acid only after periods of dialysis not exceeding 1.5 hr. After 4.5 hr. dialysis this is no longer the case.

5. The possibility that cozymase, acting as codehydrogenase, may be required at some stage of pyruvate oxidation is discussed.

6. Phosphoglycerate is oxidized by pigeon brain dispersions almost as readily as is pyruvate. This is due to its rapid conversion into pyruvate + H₃PO₄. The formation of phosphopyruvic from phosphoglyceric acid, in brain dispersions, and the transfer of phosphate from phosphoglyceric to adenylic acid, in brain extracts, have been shown to occur in the same way as in skeletal muscle.

7. Citrate or α -ketoglutarate are much less active than any of the C₄ dicarboxylic acids in catalysing the oxidation of pyruvate by brain dispersions. This is discussed.

8. The necessity of fumarate and adenylic acid for the oxidation of pyruvate by kidney cortex dispersions (rabbit) has been demonstrated in preliminary experiments.

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CCXLVI. THE ENZYMIC PHOSPHORYLATION OF VITAMIN B₁

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THE enzymic phosphorylation of vitamin B₁ has been reported by a number of investigators. In most cases these experiments were carried out with yeast cells, dried or fresh, or with sliced or minced animal tissues. Owing to the complexity of the material no clear insight into the mechanism of the phosphorylation has yet been possible. Lohmann & Schuster [1937] described the synthesis of cocarboxylase from vitamin B₁ by minced brain and intestinal mucosa; simultaneous addition of vitamin B₁, adenylyl pyrophosphoric acid, creatine phosphoric acid and phosphopyruvic acid led to an increased synthesis compared with the addition of vitamin B₁ only. Ochoa [1939], on the other hand, failed to obtain an increased synthesis with liver preparations on addition of adenylyl pyrophosphoric acid, phosphoglyceric acid or both together, and adduced this as "evidence against a direct transfer of phosphate from either compound". Lipschitz *et al.* [1938] observed a stimulating influence of hexosediphosphate on the synthesis of cocarboxylase from vitamin B₁ by washed dried yeast in the presence of boiled tissue extracts. Their results are however interpreted by Ochoa & Peters [1938] as merely meaning a removal of acetaldehyde from the reaction mixture by dismutation with triosephosphate.

For the work to be described below a cell-free protein preparation from yeast was used both as promotor and indicator of cocarboxylase synthesis. It was possible to demonstrate that phosphorylation of both vitamin B₁ and vitamin B₁-monophosphoric acid only occurs in the presence of adenylyl pyrophosphoric acid or such reactions as entail its intermediate formation. In its absence no synthesis of cocarboxylase takes place. It is to be concluded that a direct transfer of phosphate from adenylyl pyrophosphoric acid is the mechanism by which vitamin B₁ is phosphorylated.

EXPERIMENTAL

The following abbreviations will henceforth be used: vitamin B₁=B₁; vitamin B₁-monophosphoric acid=B₁-monoP; adenylyl pyrophosphoric acid=ATP.

Preparations: B₁ hydrochloride was a synthetic product from Roche Products, Ltd.

B₁-monoP was prepared from a mixture of B₁-monoP and cocarboxylase obtained by the method which was described by Weijlard & Tauber [1938] for the preparation of cocarboxylase. After acid hydrolysis the product was purified *via* the Au-salt according to Lohmann & Schuster [1937]. M.P. 197° (uncorr.). P=7.5%. The substance was free from B₁ and cocarboxylase.

Cocarboxylase was a synthetic product prepared by a new method shortly to be described. P=13.0%.

ATP was prepared from rabbit muscles by the method of Lohmann [1931]. 10.06 mg. Ba salt contained 0.920 mg. total P, 0.615 mg. pyro-P, 0.02 mg. inorganic P. Purity, 88 %.

Phosphopyruvic acid was synthesized according to Kiessling [1936] and converted into the crystalline Ag-Ba double salt. Ag-Ba salt: P split off by I_2 in alkaline solution: 6.2 %, total P, 6.2 %; inorg. P, 0. Purity, 90 %.

Muscle adenylic acid was a commercial product from Messrs Fränkel and Landau.

Methods: manometric measurements were carried out in the Warburg apparatus at 25° in air. All components were placed in the main part of the vessel with the exception of the substrate which was tipped in from the side bulb after the first reading.

Preparation of enzyme: the method of Warburg & Christian [1938] for the preparation of enzymes free from dissociable prosthetic groups has been adapted for our purpose.

50 g. of air-dried Löwenbräu bottom beer yeast are mixed with 150 ml. water and incubated at 37.5° for 3 hr. After centrifuging, the yeast residue is washed with a volume of water equal to the supernatant extract. The combined extract and washings are cooled to 0° and mixed with 1/2 vol. of cold acetone. The precipitate is discarded. 10 vol. of acetone are now added to the solution. The precipitate is allowed to settle and, after the supernatant liquid has been siphoned off, is centrifuged and dried *in vacuo*. Yield 8.5–9 g. Of this preparation, made according to Axmacher & Bergstermann [1934], a large stock can be prepared and kept in the desiccator for many months without loss of activity.

2 g. of the dry acetone precipitate are dissolved by grinding in a mortar with water and the volume is made up to 60 ml. After cooling to 0° 2 ml. 2 *N* acetic acid are added giving pH about 4.5. The precipitate is centrifuged off and rejected. 30 ml. saturated $(NH_4)_2SO_4$ are added to the solution and the precipitate is again discarded. After a further 30 ml. saturated $(NH_4)_2SO_4$ have been added the active precipitate is centrifuged and redissolved in 20 ml. *M*/20 phosphate buffer pH 6.2.

At this stage the activity of carboxylase is 5 times higher than in the original Lebedew maceration juice (Table I). For a rapid estimation of protein Folin & Ciocalteu's reagent was used, the ratio of the tyrosine content to the dry wt. of the protein having been determined preliminarily at the various stages of purification.

Table I. *Activity of carboxylase preparations*

	μ l. CO_2 /min./mg. protein	Tyrosine content of protein %
Original Lebedew extract	23	9.8
After acetone precipitation	55	12.0
After $(NH_4)_2SO_4$ precipitation	107	12.6

Owing to the increasing fragility of the enzyme it was not found expedient to attempt a higher degree of purification before the enzyme was dissociated by acidification. This process was carried out in the following manner: one-half of the solution of the $(NH_4)_2SO_4$ -precipitate (about 12 ml.) was diluted with 50 ml. water and 40 ml. sat. $(NH_4)_2SO_4$ were added producing but a faint opalescence. The mixture was cooled to –5° and 30 ml. *N*/10 HCl, 1/3 saturated with respect to $(NH_4)_2SO_4$, were added fairly rapidly from a burette while shaking vigorously. After centrifuging the precipitate was once washed with 20 ml. 3/4 saturated $(NH_4)_2SO_4$. This operation was now repeated with the second half of the enzyme.

The yield of active protein was found to be higher when the acidification was thus carried out with two separate portions than when the scale was doubled. The combined precipitates were now triturated with 15 ml. *M*/20 phosphate buffer pH 6.2 and denatured protein was centrifuged off. The protein content of the enzyme solution was in each case rapidly determined colorimetrically with Folin & Ciocalteu's reagent 0.126 mg. tyrosine indicating 1 mg. protein. More phosphate buffer was added until 1 ml. contained 1 mg. protein. The yield of active protein was usually 25–30 mg. It could be increased to 70–80 mg. if after the addition of HCl the precipitation was completed by the addition of a further 30 ml. sat. (NH₄)₂SO₄. But in this case some undissociated protein was precipitated as well, which tended to increase the blank. This step was therefore omitted in most experiments.

Usually the enzyme solution was freshly prepared for each experiment, starting with the dry acetone precipitate. The enzyme is destroyed by dialysis, by drying and even by a few hours' standing at 0°. It can however be preserved by adding an equal volume of sat. (NH₄)₂SO₄ and keeping at 0°. For use the protein precipitate is centrifuged and dissolved in an appropriate volume of phosphate buffer.

The concentration of protein in the experiments to be described was 0.33 mg./ml.

Some properties of the enzyme: pyruvic acid is decarboxylated only after addition of cocarboxylase of which about 6–7 µg./ml. are necessary for saturation (Tables II and III). 0.1 mg. Mg⁺⁺ and 0.1 mg. Mn⁺⁺/3 ml. were added as a

Table II. *Decarboxylation of pyruvic acid*

Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml.

	µl. CO ₂ in 30 min.
No addition	30
30 µg. cocarboxylase	1080

Table III. *Decarboxylation of pyruvic acid with and without added B₁*

Reaction mixture as in exp. of Table II.

	µl. CO ₂ in 15 min.	
	Without B ₁	With 10 µg. B ₁
0	21	23
2 µg. cocarboxylase	132	125
6 µg. cocarboxylase	252	265
20 µg. cocarboxylase	349	338

routine, but inorganic phosphate is not necessary for the reaction, since it proceeds just as rapidly in citrate buffer. B₁ and B₁-monoP are both inactive as coenzymes. Contrary to the findings of Ochoa [1938] and Ochoa & Peters [1938] on alkaline-washed yeast no stimulating effect of B₁ was observed even if it was added to quantities of cocarboxylase well below saturation. This at once disposes of the difficulties encountered by these authors in assessing the amount of cocarboxylase with alkaline-washed yeast in presence of B₁.

Other enzymes are present in our protein preparation besides carboxylase. One of these is Negelein's protein A [1936] which catalyses the transfer of phosphate from phosphopyruvic acid to adenylic acid and from ATP to glucose or hexosemonophosphate [Meyerhof *et al.* 1937, 1]. The addition of an active preparation of protein A prepared by Negelein's method to 1 mg. of our protein did not increase the cleavage of phosphopyruvic acid. The protein also contains

the enzyme or enzymes necessary for the transfer of phosphate from ATP to B_1 or B_1 -monoP. The latter may be identical with, or may form a part of "protein A", but this reaction, too, was in no way accelerated by the addition of protein A.

The synthesis of cocarboxylase

Though both B_1 and B_1 -monoP are quite inactive as coenzymes of carboxylase they become, after an induction period of about 15 min., very active if some ATP is added (Fig. 1). ATP alone possesses a slight coenzyme activity, a fact which will be discussed below. Since ATP does not act as a catalyst in this reaction but as a phosphate donator, the amounts required are of the order of 1-3 mg./3 ml. (Fig. 2) whereas the blank reaction of ATP alone reaches its maximum at a much lower concentration. ATP cannot be replaced by adenylic acid (Table IV).

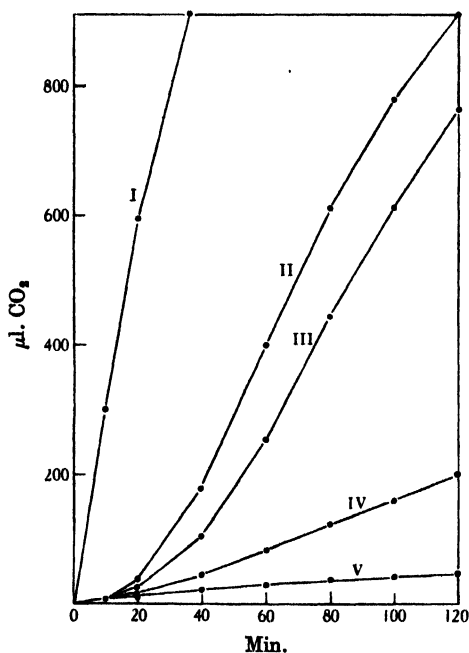


Fig. 1. Synthesis of cocarboxylase from B_1 and B_1 -monoP. Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml. I, with 30 $\mu g.$ cocarboxylase; II, with 10 $\mu g.$ B_1 + 2 mg. ATP; III, with 10 $\mu g.$ B_1 -monoP + 2 mg. ATP; IV, with 2 mg. ATP; V, with 10 $\mu g.$ B_1 .

Table IV. *Effect of adenylic acid on the decarboxylation of pyruvic acid in presence of B_1 and B_1 -monoP*

Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/50$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml.

	$\mu l. CO_2$ in 2 hr.	
	Without adenylic acid	With 0.5 mg. adenylic acid
0	58	40
30 $\mu g.$ B_1	48	50.5
60 $\mu g.$ B_1 -monoP	50	57
20 $\mu g.$ cocarboxylase (60 min.)	1068	—
0.5 mg. ATP + 30 $\mu g.$ B_1	440	—

Almost the maximum effect is obtained with a concentration of B₁ as low as 3 μ g./3 ml. It is only slightly higher with 10 μ g./3 ml. Higher concentrations are clearly inhibitory (Fig. 3).

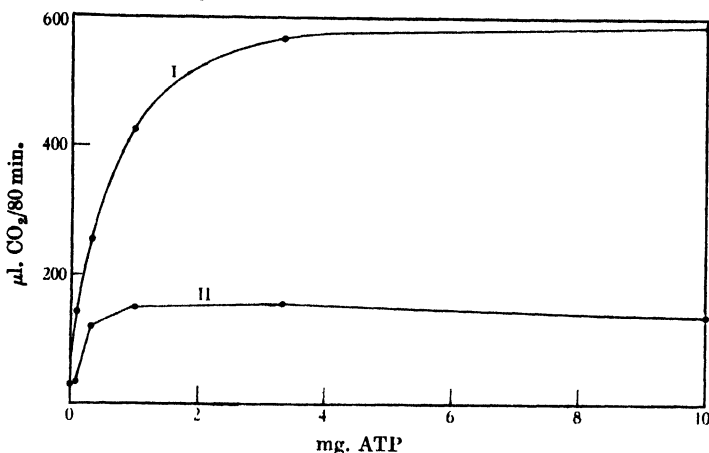


Fig. 2. Activity of ATP. The points of the curves indicate the decarboxylation of pyruvic acid after 80 min. Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml. In addition each vessel contained varying quantities of ATP. I, with 30 μ g. B₁; II, without B₁.

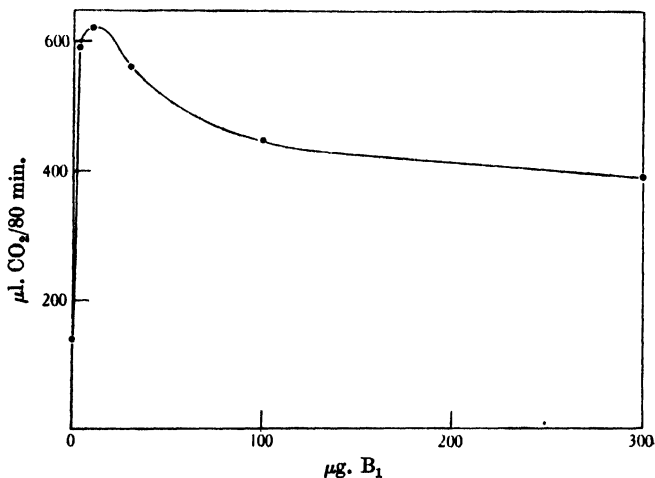


Fig. 3. Activity of B₁. The points of the curve indicate the decarboxylation of pyruvic acid after 80 min. Each vessel contained 1 mg. protein, *M*/30 phosphate buffer pH 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺, 3 mg. ATP and varying amounts of B₁. Total vol. 3 ml.

It has repeatedly been observed that with B₁-monoP the induction period is longer and the activity slightly lower than with B₁ (Fig. 1). With the latter the activity reaches about 1/3–1/2 of the initial activity of the enzyme saturated with cocarboxylase. This would correspond to the formation of 0.3–1 μ g. cocarboxylase, since with 1 μ g. cocarboxylase the rate of reaction is about half the maximal velocity (cf. Table V). But this is a minimum estimate: the highest activity is only observed after 40 min. when the activity of the enzyme may have declined. There is another fact to be considered: ATP has an inhibitory action on

carboxylase. This inhibition becomes apparent only when an enormous excess of ATP is present compared with cocarboxylase. It increases with increasing concentration of ATP and decreasing concentration of cocarboxylase and it decreases with time (Table V).

Table V. *Inhibition of carboxylase by ATP*

Exp. 1. The enzyme used for this experiment was prepared by suitable dilution from the redissolved $(\text{NH}_4)_2\text{SO}_4$ -precipitate before acidification. Each vessel contained 0.2 mg. protein, $M/30$ phosphate buffer pH 6.2, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} , $M/30$ pyruvate; total vol. 3 ml.

	$\mu\text{l. CO}_2$ in 15 min.	Inhibition %
0	248	—
0.1 mg. ATP	249	0
0.3 mg. ATP	235	5
1.0 mg. ATP	200	20

Exp. 2. Enzyme dissociated by acidification as usual. Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} ; total vol. 3 ml.

	Without ATP $\mu\text{l. CO}_2$		With 2 mg. ATP $\mu\text{l. CO}_2$		Inhibition (%)	
	First 10 min.	Second 10 min.	First 10 min.	Second 10 min.	First 10 min.	Second 10 min.
0	20	19	22	27	0	0
1 $\mu\text{g. cocarboxylase}$	121	129	72.5	98	40	24
3 $\mu\text{g. cocarboxylase}$	182	172	130	148	28.5	14
10 $\mu\text{g. cocarboxylase}$	265	198	193.5	176	27	11
30 $\mu\text{g. cocarboxylase}$	302	200	270	213	10.5	0

This inhibitory action of ATP, which is of great theoretical interest as will be shown later, may well explain the induction period during the synthesis of cocarboxylase from B_1 , but it will tend to become insignificant once full activity is reached.

Though the observed effects can hardly be explained except by a synthesis of cocarboxylase, attempts were made to obtain further evidence of the phosphorylation of B_1 by the thiochrome method. As is well known, only unphosphorylated thiochrome is soluble in *isobutyl* alcohol whereas phosphorylated thiochrome remains in the aqueous phase. After incubation of B_1 with the enzyme in presence of ATP one would expect a decrease of fluorescence in the *isobutanol* phase and the appearance of fluorescence in the aqueous phase. Though the changes observed were in the right direction they were so small that they were almost within the limits of experimental error.

Two reaction mixtures of 15 ml. each were prepared, each containing 5 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/30$ pyruvate, 0.5 mg. Mg^{++} , 0.5 mg. Mn^{++} and 50 $\mu\text{g. B}_1$. To one of them 15 mg. ATP were added. After 80 min. 260 $\mu\text{l. CO}_2$ were formed without, and 4665 $\mu\text{l. CO}_2$ with ATP. A similar experiment was performed, in which the addition of pyruvate was omitted. At the end of the incubation period 1 ml. 10 N H_2SO_4 was added to each solution and they were placed in a boiling-water bath for 5 min. After cooling and neutralization 15 mg. ATP were added to the solutions where this addition had previously been omitted. The solutions were filtered and concentrated *in vacuo* to 10 ml. whereby all acetaldehyde was removed. 5 ml. of each solution were now oxidized with 0.2 ml. 1% $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 3 ml. 15% NaOH and extracted with 13 ml. *isobutyl* alcohol. The remaining 5 ml. served as controls after treatment with 13 ml. *isobutyl* alcohol and 3 ml. 15% NaOH . The fluorescence of the aqueous

and the *isobutanol* phases was measured in the light of a mercury lamp with a photoelectric cell connected with a mirror galvanometer. The following results were obtained (corrected for the blanks):

	Deflexion in mm.	
	Aqueous phase	<i>iso</i> Butanol phase
In absence of pyruvate:		
Without ATP	0	22
With ATP	4	20
In presence of pyruvate:		
Without ATP	0	24
With ATP	5	21

It was hoped to obtain by this method information about the equilibrium between B₁ and cocarboxylase, but since the results were rather disappointing this line was not pursued further. The only conclusion which can be drawn is that the amount of cocarboxylase formed is very small, probably a few μ g. only.

Synthesis of cocarboxylase with phosphopyruvic acid as phosphate donator

Phosphopyruvic acid is not decarboxylated by the protein preparation described whether cocarboxylase, B₁-monoP or B₁ or any combination of them be present. This is of interest, since it might have been inferred from an analogy with ATP that the pyrophosphate group in cocarboxylase has a function in the mechanism of decarboxylation involving a shift of phosphate. This, however, seems unlikely.

The decomposition of phosphopyruvic acid by our protein requires the presence of (1) a phosphate carrier, adenylic acid or ATP, (2) a phosphate acceptor, e.g. glucose. The progress of the reaction was measured by the decarboxylation of the pyruvic acid formed. In this case cocarboxylase has to be added, too. Even in the presence of the complete system the reaction is slow. Addition of protein A has no effect. If cocarboxylase is replaced by B₁ decarboxylation occurs indicating the synthesis of cocarboxylase. B₁-monoP is active as well, but clearly inferior to B₁. Whereas in the former experiments with ATP as phosphate donator adenylic acid was inactive and considerable amounts of ATP were required, in the experiments with phosphopyruvic acid ATP can be replaced by adenylic acid and both are active if present in catalytic amounts. The reaction is however slower and has a longer induction period when adenylic acid is used instead of ATP (Table VI). Similar observations were made by

Table VI. *Decomposition of phosphopyruvic acid*

Each vessel contained 1 mg. protein, *M*/60 phosphate buffer pH 6.2, *M*/75 phosphopyruvate, *M*/50 glucose, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺; total vol. 3 ml.

	μ l. CO	
	In 1 hr.	In 3 hr.
0.1 mg. ATP	19	27
0.1 mg. ATP + 30 μ g. cocarboxylase	119	342
0.1 mg. ATP + 50 μ g. B ₁ -monoP	31.5	93
0.1 mg. ATP + 50 μ g. B ₁	51	126
0.1 mg. adenylic acid	10	46
0.1 mg. adenylic acid + 30 μ g. cocarboxylase	63	312
0.1 mg. adenylic acid + 50 μ g. B ₁ -monoP	10	84
0.1 mg. adenylic acid + 50 μ g. B ₁	10	108

Meyerhof *et al.* [1937, 1] who explained it by the assumption that the transfer of phosphate occurs between adenosine diphosphate and ATP rather than between adenylic acid and adenosine diphosphate.

It was not possible to replace glucose as phosphate acceptor by stoichiometric amounts of B_1 or B_1 -monoP (Table VII). This is in agreement with the observations recorded above that the synthesis of cocarboxylase stops after amounts in

Table VII. *Decomposition of phosphopyruvic acid with B_1 or B_1 -monoP as phosphate acceptors*

Each vessel contained 1 mg. protein, $M/30$ phosphate buffer pH 6.2, $M/75$ phosphopyruvate, 0.1 mg. adenylic acid, 0.1 mg. Mg^{++} and 0.1 mg. Mn^{++} . Total vol. 3 ml.

	$\mu l.$ CO_2 in 4 hr.
30 $\mu g.$ cocarboxylase	30
30 $\mu g.$ cocarboxylase + $M/75$ glucose	342
15 mg. B_1	28.5
15 mg. B_1 -monoP	38

the region of 1 $\mu g.$ have been formed and that higher concentrations of B_1 even inhibit the synthesis of cocarboxylase. An enzymic synthesis of cocarboxylase on a preparative scale is therefore not feasible. Negative results were also obtained in experiments with dialysed muscle extract arranged on the lines of the experiments described by Meyerhof *et al.* [1937, 2]. Hexosediphosphate was used as phosphate donator. Whereas creatine was highly active as phosphate acceptor both B_1 and B_1 -monoP were inactive.

If alkaline-washed yeast is used as enzyme preparation the results are essentially similar. The breakdown of phosphopyruvic acid is however much faster and no phosphate acceptor is required, no doubt because the ATP formed is broken down by an adenylyl pyrophosphatase contained in the yeast. In presence of adenylic acid, but in absence of a phosphate donator, no synthesis of cocarboxylase from B_1 occurred with alkaline-washed yeast. The results of Table VIII

Table VIII. *Decomposition of phosphopyruvic acid and synthesis of cocarboxylase by alkaline-washed yeast*

Each vessel contained 1 ml. alkaline-washed yeast prepared according to Lohmann & Schuster [1937], $M/30$ phosphate buffer pH 6.2, 0.1 mg. Mg^{++} , 0.1 mg. Mn^{++} and $M/75$ phosphopyruvate. Total vol. 3 ml.

	$\mu l.$ CO_2 in 40 min.
0	6
30 $\mu g.$ cocarboxylase	21
0.2 mg. adenylic acid	65
0.2 mg. adenylic acid + 30 $\mu g.$ cocarboxylase	365
0.2 mg. adenylic acid + 50 $\mu g.$ B_1 -monoP	123
0.2 mg. adenylic acid + 30 $\mu g.$ B_1	169

closely resemble those of Lipschitz *et al.* [1938]. The boiled tissue extract used by these authors was replaced by adenylic acid and the hexosediphosphate by phosphopyruvic acid. The objections of Ochoa & Peters [1938] do not therefore apply to these experiments. Attention may again be directed to the fact that B_1 -monoP is considerably less active than B_1 and that there is a small catalytic effect of adenylic acid alone. The latter effect does not exist in the absence of a phosphate donator.

DISCUSSION

Since the synthesis of cocarboxylase from B₁ requires the intermediation of ATP all the facts known to apply to phosphorylations of this kind also apply to this particular case. Phosphorylation by inorganic phosphate can thus be expected to occur linked up with simultaneous dismutations or oxidations involving a reduction of codehydrogenases. The role of an intact respiration stressed by Ochoa [1939] points in this direction.

The fact that the synthesis of cocarboxylase from B₁-monoP requires a longer induction period than, and does not attain the same intensity as, that from B₁ suggests that the former is not an intermediate in the formation of cocarboxylase from B₁. If this is the correct explanation, one must assume that B₁-monoP is first hydrolysed to B₁ and that this in turn combines with a pyrophosphoric group transferred *in toto* from ATP.

It is probable that under the conditions of our experiments amounts of 1 μ g. cocarboxylase or less are formed. This is in agreement with the maximum rate of decarboxylation reached, with the fact that very small quantities of B₁ suffice for optimal synthesis and with the results of the thiochrome measurements. High concentrations of B₁ inhibit the synthesis, and B₁ is therefore unsuitable as stoichiometric phosphate acceptor for phosphopyruvic acid. Since a higher synthesis of cocarboxylase cannot be forced by an increase of the concentration of B₁ or ATP, the limiting factor is probably the amount of carboxylase protein present. It seems that the synthesis of cocarboxylase stops as soon as the carboxylase present is fully or partly saturated. No preparative use can therefore be made of the reactions described.

An observation which needs some comment is the small catalytic activity of ATP alone. Three possibilities were considered: (1) the preparation of ATP contained B₁, B₁-monoP or cocarboxylase as impurity. Such an explanation was suggested by the fact that the catalytic activity of ATP only appears after an induction period similar to that observed during the synthesis of cocarboxylase from B₁. For that same reason a contamination by cocarboxylase could be dismissed. But also the possibility of a contamination with B₁ or B₁-monoP could be ruled out for the following reasons: the catalytic activity was the same with two different preparations of ATP of slightly different purity. It was also observed with a preparation of adenylic acid under conditions which entailed the intermediate formation of ATP. The thiochrome test made on a solution of 10 mg. ATP was negative. Finally, if this explanation were correct, one would expect the catalytic activity to increase with increasing amounts of ATP until it reached the order attained when B₁ was added. In fact, the maximum catalytic activity is reached with very small amounts of ATP and is always very much smaller than the effect observed in presence of B₁ or B₁-monoP.

(2) The enzyme preparation may still contain some firmly affixed B₁ or B₁-monoP. This, too, is very improbable. No fluorescence was revealed in a thiochrome test made on a solution of 8.5 mg. protein and carried out in the manner described. Furthermore, the catalytic activity of ATP did not vary substantially with different enzyme preparations.

(3) The only remaining explanation is that the catalytic effect is due to ATP itself. The constitution of ATP resembles that of cocarboxylase in two respects: it possesses a pyrophosphoric group which may enable it to attach itself to the enzyme, and it possesses, at the same position of the pyrimidine ring, a NH₂-group which, according to Langenbeck [1933], is essential to

catalysts of decarboxylation. ATP may thus be assumed to form with the active protein a "pseudo-carboxylase" of much inferior activity.

If ATP has some affinity for the carboxylase protein it would be expected to compete with cocarboxylase. This is indeed the case. The inhibition of carboxylase by ATP, which has been demonstrated, has all the properties of a competitive one. Since it becomes evident only with an excess of ATP over cocarboxylase of 1000 times or more, the affinity of ATP for the carboxylase protein is very small compared with that of cocarboxylase.

It will be recalled that Albers & Schneider [1936] in a short communication claimed a catalytic effect of adenylic acid with alkaline-washed yeast. In my experiments adenylic acid was quite inactive under these conditions unless a phosphate donator such as phosphopyruvic acid was present. The effect observed by Albers & Schneider was probably due to the presence or the formation of ATP too.

SUMMARY

A soluble yeast protein has been prepared from which cocarboxylase was removed by acidification in presence of ammonium sulphate. The addition of cocarboxylase results in a vigorous decarboxylation of pyruvic acid. Vitamin B₁ has no stimulating effect on the reaction even if added to quantities of cocarboxylase well below saturation.

Though both vitamin B₁ and vitamin B₁-monophosphate are quite inactive as coenzymes of carboxylase they become very active in presence of adenylic pyrophosphate. After about 40 min. the activity reaches 1/3-1/2 of the initial activity of the enzyme saturated with cocarboxylase.

Vitamin B₁-monophosphate was consistently found to be slightly inferior in activity to vitamin B₁. This is interpreted as meaning that the monophosphate is not an intermediary of the synthesis and must first be hydrolysed to the unphosphorylated vitamin.

Adenylic pyrophosphate alone has a small catalytic action in combination with the carboxylase protein. It is assumed that this is due to the adenylic pyrophosphate attaching itself to the enzyme and forming a "pseudo-carboxylase". In support of this a competitive inhibition was observed if adenylic pyrophosphate was added in large excess over cocarboxylase.

Phosphopyruvic acid can act as phosphate donator for the synthesis of cocarboxylase in presence of catalytic amounts of adenylic acid or adenylic pyrophosphate.

The synthesis of cocarboxylase stops when the carboxylase protein is partly or fully saturated. No preparative use can therefore be made of the reactions described.

A very liberal gift of vitamin B₁ hydrochloride was received from Messrs Roche Products, Ltd., through the courtesy of Dr F. Bergel, Research Director. A sample of phosphopyruvic acid which was used for some preliminary experiments was obtained from Dr D. M. Needham. I wish to express my sincere thanks for these donations. I also thank Dr W. Kelly for permission to use his fluorometer.

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CCXLVII. FLAVIN-ADENINE-DINUCLEOTIDE IN RAT TISSUES¹

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It now seems clear that flavin-adenine-dinucleotide acts as the coenzyme or "prosthetic group" of at least five enzymes of biological interest: (1) *D*-amino acid oxidase [Warburg & Christian, 1938], (2) the flavoprotein from milk of Corran & Green [1938] which is at all events similar to the xanthine oxidase of Ball [1939], (3) the yeast flavoprotein of Haas [1938], (4) diaphorase of Euler & Hellström [1938], or coenzyme factor of Dewan & Green [1938] (cf. also Straub [1939]; Straub *et al.* [1939]), and (5) yeast fumarate hydrogenase [Fischer *et al.* 1939]. In view of the great importance of this nucleotide it seemed of interest to determine the amount present in boiled extracts of various rat tissues under different experimental conditions.

Vivanco [1935, 1, 2] has described a lowering of the lactoflavin content of rat tissues (especially heart and liver) following the feeding of a flavin-free diet. Similar results have been reported for liver [Kuhn *et al.* 1935], and for liver and possibly heart [Groen & Schuzl, 1938]. We shall show that there is a similar decrease of the flavin-adenine-dinucleotide in heart and liver tissue.

Experimental methods

The flavin-adenine-dinucleotide was determined by the method of Warburg & Christian [1938], the O_2 utilized in the oxidative deamination of alanine being measured manometrically. The nucleotide-free enzyme was prepared from a kidney acetone powder, and partially purified as described by Negelein & Brömel [1939]. The separation of the flavin-adenine-dinucleotide was done according to Warburg & Christian's [1938] directions. In the final stage the enzyme from 35 g. acetone powder was dissolved in 7 ml. *M*/15 pyrophosphate buffer pH 8.3 and diluted with ice-cold water to 90 ml.

The acetone powder was moderately stable, and also the entire enzyme solution if kept at 0°. In absence of coenzyme solutions were only stable for one week.

To each manometric bottle were added to the main chamber: (1) 1 ml. pyrophosphate buffer pH 8.3, (2) 0.4 ml. enzyme solution, (3) 0.5 ml. solution to be estimated, or standard flavin-adenine-dinucleotide. The side bulb contained 0.2 ml. 4.5 % solution of *DL*-alanine. KOH-filter papers were placed in the central well. Air filled the gas space and the temperature was 38°. The substrate was tipped in when the bottles were placed in the bath and the O_2 uptake was

¹ A preliminary report of some of these experiments has been published in *Nature, Lond.*, 1939, 144, 787.

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measured for a 30 min. period commencing exactly 15 min. later. During this period the rate of O_2 uptake remained constant.

The relation between O_2 uptake in 30 min. and added flavin-adenine nucleotide in our experiments is shown in Fig. 1. For amounts of nucleotide less than 1 $\mu g.$ the relation between O_2 uptake and nucleotide added is almost linear.

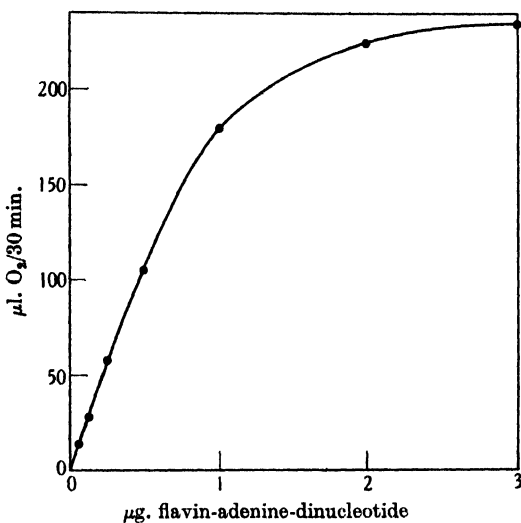


Fig. 1. The relation between O_2 uptake and flavin-adenine-dinucleotide added.

Solutions to be determined were always diluted so that the nucleotide content fell within this range. For each series of determinations at least two bottles containing known amounts of a standardized solution were used to correct for daily fluctuation in the enzyme activity.

Preparations

Flavin-adenine-dinucleotide. A sample of the pure nucleotide kindly sent by Prof. O. Warburg to Prof. R. A. Peters.

Vitamin B₁ hydrochloride. Synthetic specimens kindly supplied by Messrs Hoffmann La Roche and Messrs Bayer.

Lactoflavin and alanine. These were commercial preparations. The lactoflavin was flavin-adenine-dinucleotide-free.

1. The flavin-adenine-dinucleotide content of boiled extracts of tissues from normal rats

The rats, males from the usual laboratory stock, were fed on a synthetic diet supplemented with whole yeast and cod liver oil. They were killed by decapitation, bled, the tissues weighed in a weighing bottle, finely minced with scissors and ground with 5 volumes distilled water in a small porcelain mortar. The tissue suspension was then plunged into a water bath at 80° for 15 min., cooled, centrifuged and suitably diluted. Table I gives the average values found. The individual values are given in Table VIII of the appendix.

Table I. *Mean flavin-adenine-dinucleotide content ($\mu\text{g./g.}$ fresh tissue) in tissues of normal and adrenalectomized rats*

Condition of animal	No. of animals	Tissue	Mean	$2 \times \text{s.e. mean}$
Normal	6	Brain	10.0	± 0.8
	6	Heart	64.5	± 20.8
	6	Kidney	61.0	± 11.2
	8	Liver	77.3	± 11.4
Adrenalectomized	6	Brain	10.3	± 0.6
	5	Heart	69.4	± 15.8
	6	Kidney	58.7	± 7.4
	6	Liver	75.5	± 10.2

2. *Flavin-adenine-dinucleotide content of boiled extracts of tissues of rats on a diet deficient in flavin*

Two groups of male rats (average weight 35 g.) were fed on the following diet:

Rice starch	70
Casein (alcohol-extracted)	20
Salt mixture	5
Agar-agar	2
Cod liver oil	3

The diet was supplemented by the following per rat per day:

10 $\mu\text{g.}$ crystalline vitamin B_1 hydrochloride;
 0.2 ml. 50 % acid-alcohol yeast concentrate;
 0.2 ml. liver filtrate factor.

The yeast concentrate was prepared as described by Kinnersley *et al.* [1933], and the liver filtrate was previously treated with fuller's earth and franconite. Both were prepared by Mr L. A. Stocken, to whom our sincere thanks are due. This diet is reasonably free from flavin, especially if care is taken with the extraction of the casein.

Group A (Fig. 2) fed on this diet increased in weight but little, while group B, which received in addition a daily supplement of 50 $\mu\text{g.}$ lactoflavin per animal, showed a normal growth rate. After 30 days the animals were killed and the flavin-adenine-dinucleotide determined as before (Table II). The individual

Table II. *Mean flavin-adenine-dinucleotide content ($\mu\text{g./g.}$ fresh tissue) of rat tissue*

Group	Diet	No. of animals	Mean wt. change (g. $\pm 2 \times \text{s.e. mean}$)	Tissue	Mean $\pm 2 \times \text{s.e. mean}$
A	Flavin-deficient	6	14.9 ± 4.0	Brain	10.3 ± 0.4
				Heart*	38.2 ± 3.6
				Kidney	72.5 ± 10.8
				Liver*	41.4 ± 6.6
B	Flavin-deficient plus 50 $\mu\text{g.}$ lactoflavin per day	6	79.8 ± 14.0	Brain	12.8 ± 2.2
				Heart	92.7 ± 17.8
				Kidney	78.4 ± 15.4
				Liver	87.0 ± 22.2

* Heart and liver figures are mean for 9 animals.

results are given in the appendix (Tables IX and X). It is seen that the mean nucleotide content of the tissues of deficient rats is lower than that of those

receiving adequate amounts of flavin. This is significantly so in the case of heart and liver tissue. Application of Fisher's "*t*" test to the difference of means of the heart and liver figures gives $t=7.28$ and 4.65 respectively. For $P=0.01$, $t=3.01$. The figures are thus clearly significant. The results are represented graphically in Fig. 3.

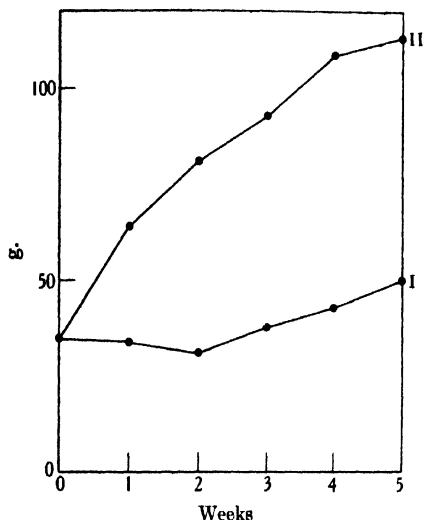


Fig. 2.

Fig. 2. Changes in weight of rats on flavin-deficient diet. Each curve = average for 6 rats. Curve I: diet only. Curve II: diet plus 50 µg. lactoflavin per day.

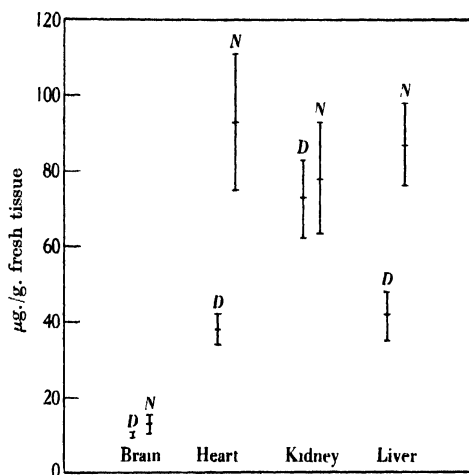


Fig. 3.

Fig. 3. Flavin-adenine-dinucleotide (µg./g. fresh tissue) in rat tissue. D, flavin-deficient diet. N, normal (i.e. flavin-deficient diet plus 50 µg. lactoflavin per day). Ordinate; mean $\pm 2 \times$ s.e. mean.

Some animals on the flavin-deficient diet developed a dermatitis, especially around the eyes, and others (results not included in the tables) died before the conclusion of the experiment. The boiled extracts from the organs of these animals still contained appreciable amounts of the nucleotide.

3. The *in vivo* synthesis of flavin-adenine-dinucleotide from lactoflavin

The flavin-adenine-dinucleotide content of liver and heart tissues of rats on a flavin deficient diet can be increased (Table III) by injection of lactoflavin. After injection of 1 mg. lactoflavin on each of three occasions, 24, 4 and $\frac{1}{2}$ hr. before killing, the nucleotide content of these organs approximates to normal values. This constitutes an *in vivo* synthesis of the nucleotide from lactoflavin.

In animals killed $\frac{1}{2}$ hr. after the first injection, however, a rise was observed in the nucleotide content of the liver, but not in the heart. This seems to indicate that the liver is able to synthesize the flavin-adenine-dinucleotide more readily than the heart. It will be seen from the table that the simultaneous injection of adenylic acid had practically no effect on the nucleotide content of the tissues. In the kidney and brain tissues of deficient rats, in which the flavin-adenine-dinucleotide content is practically normal, no further increase was observed after flavin injection.

Table III. *Flavin-adenine-dinucleotide content ($\mu\text{g./g.}$ fresh tissue) of tissue from rats on flavin-deficient diet, before and after injection of lactoflavin and adenylic acid*

Treatment of flavin-deficient animals	Liver	Heart
Mean $\pm 2 \times \text{s.e.}$ 9 uninjected animals	41.4 \pm 6.6	38.2 \pm 3.6
Injected 1 mg. lactoflavin $\frac{1}{2}$ hr. before death	58 59	40.5 42
Injected 1 mg. lactoflavin + 1 mg. adenylic acid $\frac{1}{2}$ hr. before death	58 62	38 —
Injected 1 mg. lactoflavin on each of three occasions, 24, 4 and $\frac{1}{2}$ hr. before death	67 68 57	58 62 62
Injected 1 mg. lactoflavin + 1 mg. adenylic acid on each of three occasions, 24, 4 and $\frac{1}{2}$ hr. before death	69 65	53 59

4. *Flavin-adenine-dinucleotide in boiled extracts from tissues of adrenalectomized rats*

Verzár *et al.* [1937] reported a diminution in bound (non-dialysable) and an increase in free (dialysable) flavin in the liver of rats following adrenalectomy. Later, Laszt & Verzár [1938] obtained similar results for liver, kidney and heart tissue of the cat. Since the flavin-adenine-dinucleotide probably represents the greater part of the non-dialysable flavin in liver tissue it seemed of interest to determine the amount of the nucleotide present in the tissues of rats suffering from adrenal cortical insufficiency.

The adrenals were removed from male rats by the technique described by Firor & Grollman [1932], under ether anaesthesia. The rats were of the same age-

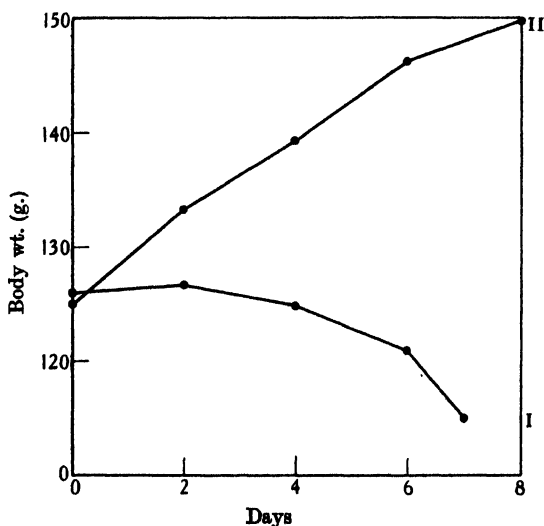


Fig. 4. Changes in weight of rats following adrenalectomy. Each curve = average for 6 rats. Curve I: adrenalectomized at day 0. Curve II: normal controls.

group and received the same diet as those described in section 1. The animals were killed when they were "in extremis" showing the typical signs—extreme weakness, loss of appetite, low body temperature, and loss of body weight (Fig. 4)—

of adrenal cortical insufficiency. Table I shows that there was no significant decrease in the flavin-adenine-dinucleotide content of the tissues of such animals. Individual values are given in the appendix (Table XI).

5. *Flavin-adenine-dinucleotide in blood*

The nucleotide content of three samples of ox blood is given in Table IV. It is seen that there is no measurable activity in unheated blood. Heating for 3 min. at 100° renders the blood active. This observation is similar to that of Goodhart & Sinclair [1939] for the cocarboxylase activity of blood, and can

Table IV. *Flavin-adenine-dinucleotide in ox blood (μg./100 ml.)*

Whole blood (unheated)	Whole blood (heated at 100° for 3 min.)	Plasma (unheated)	Plasma (heated at 100° for 3 min.)
0	55	0	22.5
0	55	0	25.5
2	49	0	17

possibly be interpreted in the same fashion, viz. the liberation of an active substance from a protein complex. They found, however, that the cocarboxylase activity was limited to the cells. Our observations show that the blood plasma contains some flavin-adenine-dinucleotide. The temperature, time and pH of heating were all found to be important. The usual practice was to heat the blood at 100° for 3 min. at pH 7. The fact that in the presence of alanine no O₂ is absorbed by unheated blood or plasma shows that it has no amino-acid oxidase activity.

Table V. *Flavin-adenine-dinucleotide in rat blood (μg./100 ml.)*

Normal	Flavin deficient	Flavin-deficient after injections of 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before death
64.5	52.5	61
56	48	61.5
64.5	52.5	—
63.5	—	—
65	—	—

Table V gives the flavin-adenine-dinucleotide values for the blood of 5 normal, 3 deficient, and 2 deficient rats which had been injected with 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before killing. The figures suggest a diminution in nucleotide content of the blood in flavin deficiency, and that this deficiency is rapidly (within 24 hr.) made good on injecting flavin.

6. *The enzymic breakdown of flavin-adenine-dinucleotide*

Incubation of tissue (usually liver) from normal and flavin-deficient animals in the presence of flavin and adenylic acid never showed a synthesis of the nucleotide; in fact, there was usually a breakdown of the nucleotide already present in the tissue. This breakdown is enzymic and Table VI shows it to be dependent on the pH of the medium.

Table VI. *Flavin-adenine-dinucleotide in liver slices (μg./g. fresh tissue) after incubation for 3 hr. at various pH. Temp. 38°*

No incubation	pH 6	pH 7.3	pH 8.4
53	40.5	32.5	20
45	41	19	10
46	38	30	9.5

Table VII shows the relative rates of nucleotide breakdown with "dispersion" preparations from brain and liver tissue after aerobic incubation at 38° and pH 8.3. It is seen that in the brain there is no significant breakdown after 3 hr. incubation, but that in the liver the breakdown is much more rapid. This is similar to the observations of Ochoa [1939] on breakdown of cocarboxylase.

Table VII. *Relative breakdown of flavin-adenine-dinucleotide ($\mu\text{g./g. fresh tissue}$) in suspensions of brain and liver tissue. pH 8.4. Temp. 38°*

Tissue	No incubation	$\frac{1}{2}$ hr.	1 hr.	2 hr.	3 hr.
Brain	12.5	12	12	12	12
	11.5	11	11	11	10.5
Liver	59	57	51	49	42
	72	67	61	56	47

DISCUSSION

The figures given for the flavin-adenine-dinucleotide content of normal rat tissues are of the same order as those of Warburg & Christian [1938]. If the mol. wt. of the nucleotide is taken as 785 and that of lactoflavin as 376, the results, molecule for molecule, are also of the same order as those reported for the lactoflavin content of rat tissues by Vivanco [1935, 1, 2] and a little higher than those of Kuhn *et al.* [1935], Verzář *et al.* [1937], Gourévitch [1937] and Groen & Schuzl [1938]. This would indicate that probably the major portion, and possibly all, of the flavin so measured was in the form of the nucleotide.

Verzář *et al.* [1937] explain their observations on adrenalectomized animals by suggesting that in adrenal cortical insufficiency there is a diminution in the proportion of the total flavin which is present in the phosphorylated form. The fact that the flavin-adenine-dinucleotide (a phosphorylated form of flavin) seems to account for most of the flavin present, and is not diminished after adrenalectomy, is difficult to correlate with the interpretation of Verzář *et al.* although it is consistent with our observations on cocarboxylase [Ochoa & Rossiter, 1939].

The decrease of the flavin-adenine-dinucleotide in the liver following a flavin-deficient diet is parallel to the decrease of cocarboxylase following a vitamin B₁-deficient diet [Ochoa & Peters, 1938], and to the decrease in cozymase [Axelrod & Elvehjem, 1939] and of V-factor [Kohn *et al.* 1939] after feeding a diet deficient in nicotinic acid. With cocarboxylase there is a general decrease in all tissues, but in the case of V-factor the decrease was observed in liver and skeletal muscle only. Similarly, the decrease in flavin-adenine-dinucleotide was observed only in liver and heart (skeletal muscle was not examined). This would be consistent with the view that the liver acts as a store, at the expense of which the levels in the other tissues are maintained. It is of interest to note that Groen & Schuzl [1938] obtained a diminution in O₂ uptake with liver tissue from rats on a flavin-deficient diet, and that Axelrod *et al.* [1939] have recently reported a similar decrease in O₂ uptake of liver and kidney tissue with alanine as substrate, suggesting a diminution of the flavin-adenine-dinucleotide in these tissues. The decrease in the nucleotide content of blood, paralleled by the decrease in cocarboxylase in pigeon's blood [Goodhart & Sinclair, 1939], and of the V-factor in the blood of pellagrins [Vilter *et al.* 1939], is also of interest. Kohn *et al.* [1939 for references], however, found no such decrease in the V-factor in the blood of pellagrins or of dogs suffering from acute blacktongue.

The results reported here afford a further example of a deficiency of a vitamin of the B-complex affecting important biological oxidation systems.

SUMMARY

1. The flavin-adenine-dinucleotide content of boiled extracts of brain, heart, kidney and liver tissue of normal rats has been determined.

2. There is a decrease in the flavin-adenine-dinucleotide content of boiled extracts of liver and heart tissue from rats fed on a flavin-deficient diet. No significant difference could be detected in extracts of brain and kidney tissue.

3. Injection of 1 mg. lactoflavin on each of three occasions, 24, 4 and $\frac{1}{2}$ hr. before death restores the flavin-adenine-dinucleotide content of liver and kidney tissue of flavin-deficient rats to normal levels. This constitutes an *in vivo* synthesis of the nucleotide from lactoflavin. The restoration is more rapid in the liver than in the heart.

4. There is no significant difference between the flavin-adenine-dinucleotide contents of boiled extracts of tissues from normal and adrenalectomized rats.

5. There is no flavin-adenine-dinucleotide activity in unheated blood (ox, rat), but this activity is present after heating the blood at 100° for 3 min. at pH 7. The flavin-adenine-dinucleotide in the blood of flavin-deficient rats is lower than that of normal animals, but is restored by injection of 1 mg. lactoflavin on each of three occasions, 24, 4, $\frac{1}{2}$ hr. before death.

6. The enzymic breakdown of the flavin-adenine-dinucleotide in the tissues was found to be dependent upon pH. Breakdown is more rapid with liver than with brain tissue.

We are greatly indebted to Prof. R. A. Peters for his continued interest and encouragement throughout this work.

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APPENDIX

Table VIII. *Flavin-adenine-dinucleotide in tissues of normal rats ($\mu\text{g./g. fresh tissue}$)*

Brain	Heart	Kidney	Liver
9.5	69	55	78
8.5	78	56	78
10	45	55	51.5
9	106	89	97
11	46	59	72
10.5	43	51.5	58
—	—	—	94
—	—	—	89

Table IX. *Flavin-adenine-dinucleotide in tissues of rats on flavin-deficient diet ($\mu\text{g./g. fresh tissue}$)*

Brain	Heart	Kidney	Liver
11	31	61	28
9.5	30.5	70	36.5
11	36.5	69	36
10	40	86	40.5
10	47	91	64
9.5	40.5	58	37.5
—	43	—	42
—	35	—	40
—	39	—	47

Table X. *Flavin-adenine-dinucleotide in tissues of rats on flavin-deficient diet plus 50 µg. lactoflavin per day (µg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
10	52	51.5	56
8.5	89	84	62
15	115	83	107
14	94	92	80
12.5	100	—	89
15.5	106	81	128

Table XI. *Flavin-adenine-dinucleotide in tissues of adrenalectomized rats (µg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
10	64	55	64
9	97	75	97
11	66	58	73
10.5	—	56	66
11	62	61	69
9.5	58	47	84

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CCXLVIII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE

XVIII. THE ACTION OF GUANIDINES AND AMIDINES ON THE PASTEUR EFFECT

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AMONG the quaternary N compounds previously found to inhibit the Pasteur effect in animal tissues [Dickens, 1936] were representatives of specific anti-septics, particularly derivatives of quinoline, acridine and phenazine containing basic substituents. I am indebted to Sir Henry Dale for the suggestion that it might be of interest to study the metabolic action of the powerfully trypanocidal guanidine and amidine derivatives recently described by King *et al.* [1937], and to Dr Harold King for kindly supplying me with these compounds. In the following paper it is shown that among them are some of the most powerful inhibitors of the Pasteur effect.

Despite the interest attaching to guanidine in its possible connexion with tetany, observations of its effect on tissue metabolism are few. Meyerhof [1921] found that in minced frog muscle guanidine in concentration of $M/75$ and above caused a slight fall of respiration, below $M/100$ sometimes a slight increase. Banu & Gavrilesco [1934] investigated the action of guanidine and methyl-guanidine salts on the respiration of brei of pigeon brain, rat liver and muscle, and rabbit blood and nerve. They found in all cases a small inhibition of respiration; the concentration of inhibitor used (*c.* $M/100$, or more) was so high, however, that their postulated connexion of this effect with tetany appears questionable. The conditions of their experiments precluded the observation of the main effect of guanidine on brain metabolism reported here.

The only previous observations on the effect of guanidine on the Pasteur effect are those of Mendel, which according to Bumm & Appel [1932] were mentioned at a discussion of the Kaiser Wilhelm Institut für medizinische Forschung at Heidelberg in 1932: "Warburg fand z.B. den Blausäureäthylester wirksam" (on the Pasteur effect), "Mendel das Guanidin, wir wollen hinzufügen das auch Arginin die Pasteursche Reaktion unter Umständen partiell hemmen kann" [Bumm & Appel, 1932]. In a personal communication, Dr Mendel kindly informed me of his findings, several of which are confirmed and extended in the present paper: I was unable, however, under the same conditions to observe the effect of arginine mentioned by Bumm & Appel.

EXPERIMENTAL

All experiments were made at 37.5° in the Warburg apparatus, using tissue slices suspended in NaHCO_3 -Ringer solution containing 0.2% glucose and in equilibrium with $\text{O}_2 + 5\% \text{CO}_2$ or $\text{N}_2 + 5\% \text{CO}_2$. The tissue used was rat brain cortex unless otherwise stated. In order to follow the time course the Warburg 2-vessel method was used for all aerobic experiments. "0 min." was 15 min. after inserting the vessels in the thermostat.

Where necessary the added substances (usually hydrochlorides) were neutralized before use; generally the dilutions were high enough for this to be immaterial, and for the same reason retention of CO_2 by the medium was negligible. However, with extremely high dilutions ($10^{-6} M$) a technical difficulty not previously experienced became evident; the absolute amount of inhibitor contained in the vessel with the smaller volume of solution was evidently insufficient to saturate the tissue, while in the larger volume normal inhibition occurred. This indicates either absorption or chemical reaction of the guanidine with some tissue component; it was avoided to some extent by taking tissue weights approximately in proportion to the liquid volumes. This also has the advantage of giving similar amounts of lactic acid per ml. Ringer solution in the two vessels. The effect of this phenomenon would be to give false low values for respiration and aerobic glycolysis, hence it could not have entered significantly with concentrations higher than about $10^{-6} M$, which caused increased respiration and glycolysis.

Guanidine. Guanidine carbonate (A.R., British Drug Houses) was dissolved directly in the Ringer solution. (The term "molar" refers throughout to the content of guanidine or other bases.) Addition of guanidine salt in $10^{-3} M$ concentration completely inhibits the Pasteur effect in rat brain slices suspended in Ringer solution or serum (Table I). The respiration is unchanged or increased; the aerobic glycolysis is greatly increased and may exceed the anaerobic glycolysis

Table I. *Action of guanidine on metabolism of rat brain cortex*

Guanidine	Time (min.)	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Medium: Ringer solution				
Nil	0-120	12.0	1.5	15
$10^{-3} M$	0-30	17.9	26.4	—
	30-60	16.3	26.8	—
	60-90	20.5	26.2	—
	90-120	18.9	22.0	—
$5 \times 10^{-5} M$	0-30	11.9	4.8	—

(Anaerobic glycolysis with $10^{-3} M$ guanidine present from start; 0-60 min. 125%, 60-120 min. 140% of control without guanidine.)

Medium: inactivated horse serum				
Nil	0-60	10.6	3.0	—
$10^{-3} M$	0-30	12.7	15.7	—
	30-60	13.4	17.3	—

Lactic acid estimation: Ringer solution, $O_2 + CO_2$. Chemical estimation by method of Friedemann & Kendall, after $\text{Cu-Ca}(\text{OH})_2$ treatment. Duration 100 min. (Owing to large weight of tissue relative to NaHCO_3 in the experiment with guanidine the rate of aerobic acid formation fell off during the experiment.) Simultaneous manometric observations were used to calculate total acid formation of the same tissue.

		Controls		With guanidine 10 ⁻³ M
Tissue dry wt. (mg.)	...	8.56	16.19	14.36
Chemical estimations:				
Lactic acid, final (mg.)		0.322	0.542	1.202
Lactic acid, preformed (mg.)		0.057	0.107	0.135
Lactic acid formed (mg.)		0.265	0.435	1.067
Manometric estimations:				
Total acid formed, calc. as lactic (mg.)		0.248	0.469	1.04
Chemical				
Manometric × 100		106 %	93 %	98 %

of the control; the anaerobic glycolysis is often at first increased but later falls slowly. At a concentration of $5 \times 10^{-5} M$ guanidine has no appreciable effect. Chemical estimation of the aerobic lactic acid shows that it accounts for practically the whole aerobic acid formation (Table I).

Table II. *Reversal of guanidine action*

Rat brain cortex. $O_2 + CO_2$. $NaHCO_3$ -glucose-Ringer solution. 37.5° . Guanidine conc. $10^{-3} M$.

Time min.	Medium	Q_{O_2}	$Q_G^{O_2}$	Medium	Q_{O_2}	$Q_G^{O_2}$
0-20	Guanidine-Ringer (Transferred to fresh Ringer)	19.2	18.3	Plain Ringer	15.1	2.8
40-60	Plain Ringer	11.0	6.3	"	(Continued)	
60-75		13.5	5.6		13.7	2.5
75-90		12.5	4.1		9.9	2.5
90-110		11.6	1.9		7.5	2.4

(Control with $10^{-3} M$ guanidine present throughout: $Q_G^{O_2}$ mean over 2 hr. = 18.2.

Table III. *Action of guanidine on various cells*

Medium: animal tissues, Ringer- $NaHCO_3$ -glucose; yeast, $M/20$ KH_2PO_4 -glucose.

	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Rat amnion:			
Guanidine $10^{-3} M$	8.6	6.1	12.6
Control	6.4	5.7	11.6
Guinea-pig medulla of kidney:			
Guanidine $10^{-3} M$	7.6	12.5	30.0
Control	9.5	8.4	25.8
Jensen rat sarcoma:			
0-30 min. no guanidine	9.0	28.6	31.3
At 30 min. tipped in guanidine from side bulb to make $10^{-3} M$:			
40-60 min. with guanidine	7.1	26.2	31.3
Baker's yeast:			
Guanidine $M/500$	51.4	42.7	133
Control	55.5	20.4	150

The action of guanidine on rat brain is reversed merely by transferring the tissue to guanidine-free Ringer solution (Table II). The reversal is not instantaneous but is complete about 50 min. after transference. This suggests a slow dissociation of some complex formed by the guanidine; it can hardly be merely a slow diffusion out of the tissue, for the inward path is rapid as shown by the rapid inhibition of the Pasteur effect which occurs within a few minutes of the addition of guanidine.

These effects of guanidine are absent or less marked in other tissues tested (Table III).

Methylguanidine, as-dimethylguanidine, agmatine. Methylguanidine HCl (B.D.H.) acts like guanidine, *as*-dimethyl guanidine HCl (Roche) shows only slight activity in inhibiting the Pasteur effect, and both substances poison the respiration slowly. Agmatine (δ -aminobutylguanidine), used as the sulphate (Roche), differed from the other compounds studied in that the aerobic glycolysis steadily increased during the experimental period of $2\frac{1}{2}$ hr. (Table IV). It is of interest that arginine, which yields agmatine on decarboxylation, did not inhibit the Pasteur effect.

Table IV. *Methylguanidine, as-dimethylguanidine, agmatine*Rat brain cortex. NaHCO_3 -Ringer-glucose. $\text{O}_2 + \text{CO}_2$. 37.5° . Tissue from 2 rats mixed.

Time (min.)	Q_{O_2}	$\text{Q}_{\text{O}_2}^{\text{O}_2}$	Q_{O_2}	$\text{Q}_{\text{O}_2}^{\text{O}_2}$
	Control (no addn.)		<i>as</i> -Dimethylguanidine $10^{-3} M$	
0- 60	12.8	2.4	12.1	6.5
70- 90	11.9	2.0	5.3	7.8
90-120	13.3	1.6	4.9	4.1
	Methylguanidine $10^{-3} M$		Agmatine sulphate $10^{-3} M$	
0- 60	11.1	19.3	13.8	5.8
70- 90	8.8	21.0	15.0	8.0
90-120	5.8	16.6	13.2	10.3

Table V. *Guanido-derivatives, biuret, urea and acetamide*Rat brain cortex. NaHCO_3 -Ringer-glucose. $\text{O}_2 + \text{CO}_2$. 37.5° .

Time (min.)	Q_{O_2}	$\text{Q}_{\text{O}_2}^{\text{O}_2}$	Q_{O_2}	$\text{Q}_{\text{O}_2}^{\text{O}_2}$	Q_{O_2}	$\text{Q}_{\text{O}_2}^{\text{O}_2}$
	Control (no addn.)		Creatine $10^{-3} M$		Arginine $10^{-3} M$	
0- 30	11.6	1.9	5.9	2.7	6.9	2.4
30- 60	11.1	0.3	3.2	0	2.8	0.0
60-120	11.1	0.6	1.9	0.1	1.0	0.0
	Control (no addn.)		Glycocyamine $10^{-3} M$		Biuret $10^{-3} M$	
0- 30	15.8	5.0	13.3	4.8	12.4	1.5
30- 60	14.9	2.5	7.9	2.2	12.3	-0.2
60-120	10.2	2.6	6.0	2.3	11.3	0.1
	Control (no addn.)		Acetamide $M/50$		Urea $M/50$	
0- 20	12.1	4.4	11.7	3.3	12.9	3.2
20- 60	11.9	2.8	11.2	1.6	13.0	3.2

Inactive compounds. Among compounds of similar structure to the above, the guanido-derivatives arginine, creatine and glycocyamine were all rather surprisingly toxic to the respiration without any inhibition of the Pasteur effect such as is shown by guanidine itself. All the above were tested in $10^{-3} M$ solution. At the same concentration biuret, and in $M/50$ solution urea and acetamide, had no effect whatever (Table V).

Decamethylenediguanidine and 1:11-undecanediamidine. These two substances, used as dihydrochlorides, showed quite remarkable activity in inhibiting the Pasteur effect (Table VI). With the higher concentrations (8×10^{-4} and $5 \times 10^{-5} M$) the metabolism became very much increased and unstable, particularly the aerobic glycolysis which rose to an unusual height for the tissue. This effect is reminiscent of that of disturbance of the ionic balance of the medium [Dickens & Greville, 1935] with the difference that the increase extends even to the anaerobic glycolysis, which always falls below normal when salt effects are the cause of aerobic glycolysis. Whatever may be the explanation of the guanidine effect, it cannot very well be due to a different permeability aerobically and anaerobically, such as has been assumed to be the basis of the salt effect.

For the reason mentioned in the introduction to the experimental part, the upper limit of dilution which is active is difficult to determine. $10^{-6} M$ solutions were sometimes active, sometimes not. One factor is undoubtedly the ratio weight of tissue : weight of guanidine or amidine compound (Table VI). For a substance of mol. wt. 330, a $10^{-6} M$ solution contains $0.33 \mu\text{g.}$ per ml. or 1 part per 3 million. This amount is of the same order as that of vitamin B_1 required to produce the catatorulin effect in avitaminous pigeon brain [Peters, 1937]. It is

Table VI. *Decamethylenediguanidine (Synthalin), 1:11-undecanediamidine, etc.*

Rat brain cortex. NaHCO_3 -Ringer-glucose. 37.5° . Zero time=15 min. after putting in the bath.

Substance	Conc. <i>M</i>	Time min.	With substance			Control		
			Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Decamethylene- diguanidine	8×10^{-4}	0-30	22.6	37.6	—	13.3	4.3	—
		30-60	16.6	25.5	—	11.4	1.2	—
		60-90	12.6	18.8	—	10.7	1.8	—
		90-120	7.2	14.6	—	10.9	0.5	—
	5×10^{-5}	0-20	16.2	25.2	23.0	15.7	1.5	13.4
		20-40	12.6	26.1	20.6	15.6	0.7	12.6
		40-60	10.3	27.2	20.0	15.6	0.4	12.3
		60-80	8.5	10.7	18.2	13.9	0.3	11.8
	10^{-6} (7 mg. dry wt. tissue)	0-90	(12.0)*	22.1	—	13.4	2.0	—
	10^{-6} (14 mg. dry wt. tissue)	0-120	14.8	2.8	—	10.8	1.2	14.2
1:11-Undecane- diamidine	8×10^{-4}	0-60	2.4	22.3	—	12.3	2.7	—
		60-120	1.9	15.0	—	10.8	1.1	—
	5×10^{-5}	0-60	10.4	20.3	15.7	9.8	1.1	15.4
		60-120	11.0	21.5	13.8	9.7	1.3	13.1
Decamethylene- diisothiurea	8×10^{-4}	0-60	(12.0)*	25.0	—	9.8	1.1	15.4
		60-120	16.8	12.0	—	12.3	2.7	—
	5×10^{-5}	0-60	7.8	3.9	—	10.8	1.1	—
		90-120	9.6	9.6	—	9.5	0.9	13.2
Hexadecamethylene- diguanidine	Satd.	0-60	12.6	4.4	—	15.6	1.2	12.8
	(< 4×10^{-4})	60-80	8.4	3.9	—	13.9	0.3	11.8

* Approx. only with 10^{-6} *M* conc.: see text.

evident, therefore, that the effects described in this paper are catalytic or anti-catalytic.

King *et al.* [1937] found that the trypanocidal activity *in vivo* of polymethylene diguanidines increased with the length of the C-chain, being low for the 2 to 6-C members, becoming first apparent with the octamethylenediguanidine and increasing in the 10 to 14-C compounds. It is of interest to note that on the Pasteur effect guanidine itself has only about 1/1000th of the activity of decamethylenediguanidine. The only higher member of the series tested, hexadecamethylenediguanidine, was however almost insoluble in the saline medium used, being only partially dissolved when added in 4×10^{-4} *M* concentration; consequently the actual amount present in solution was unknown. Under these conditions it increased aerobic glycolysis but slightly (Table VI).

Decamethylenediisothiurea, found by King *et al.* [1937] to be trypanocidal only *in vitro*, showed a fairly well-marked guanidine effect in 5×10^{-5} *M* solution: in higher concentration (8×10^{-4} *M*), after an initial effect it proved toxic to both respiration and glycolysis. This compound was used as the dihydrobromide (Table VI).

Inactive compounds related to the above group. Acetamide itself in 10^{-3} *M* solution poisons both respiration and glycolysis: in lower concentration (10^{-5} *M*) it is without effect (Table VII). Here again the presence of the long-chain substituent appears necessary both for trypanocidal activity [King *et al.* 1937] and for inhibition of the Pasteur effect by high dilutions.

2:7-Naphthalenediamidine and *pp'*-diguandodiphenylmethane were also inactive in 5×10^{-5} *M* concentration (Table VII); here the incorporation of the polycarbon chain in aromatic ring systems appears to destroy activity, but it is possible that this might be due to inability to permeate the cell.

Table VII. *Inactive compounds related to those of Table VI*

Substance	Conc. <i>M</i>	Time min.	With substance		Control	
			Q_{O_2}	$Q_{O_2}^{O_2}$	Q_{O_2}	$Q_{O_2}^{O_2}$
Acetamidine	10^{-3}	0-30	5.5	3.2	16.9	3.8
		30-60	3.6	1.1	15.1	2.1
	10^{-5}	0-60	16.1	3.4	16.0	3.5
2:7-Naphthalenediamidine	5×10^{-5}	0-120	14.3	2.4	14.3	3.7
<i>pp'</i> -Diguandodiphenylmethane	5×10^{-5}	0-120	12.3	2.8	14.3	3.7

Table VIII. *Various trypanocidal substances and related compounds*

Substance	Conc. <i>M</i>	Time min.	With substance		Control	
			Q_{O_2}	$Q_{O_2}^{O_2}$	Q_{O_2}	$Q_{O_2}^{O_2}$
Germanin (Bayer 205)	10^{-4}	0-120	10.9	2.1	11.1	1.5
Trypan blue	10^{-3}	0-30	11.1	4.0	12.6	2.9
		30-60	10.3	1.6	10.6	2.1
Rivanol	5×10^{-5}	0-30	9.4	4.8	As above	
		30-60	5.8	4.4		
Gonacrine (diaminoacridine methochloride)	5×10^{-5}	0-120	17.7	21.6	14.3	3.4
<i>p</i> -Aminophenyl-dichloroarsine	10^{-3}	0-60	0.1	0.2	11.6	2.5
Picric acid	10^{-3}	0-60	11.8	0.6	11.6	2.5
Dinitro- <i>o</i> -cresol (same tissue as 1st exp. of Table I)	5×10^{-5}	0-20	24.7	9.0	11.8	2.8
		20-40	17.0	10.4	12.1	2.4
		40-60	7.4	6.1	11.5	0.9

Trypanocides not chemically related to guanidine. In addition to the above two compounds, a number of well-known trypanocides (Table VIII) proved to have no effect on brain metabolism. Thus Trypan blue (formed by coupling diazotized *o*-toluidine and H-acid), picric acid (10^{-3} *M*) and Rivanol (5×10^{-5} *M*) were quite without action, while *p*-aminophenyldichloroarsine (10^{-3} *M*) had an almost instantaneous and complete poisoning effect.

Similarly Germanin (Bayer 205) in *c.* 10^{-4} *M* concentration had no effect on metabolism over 2 hr. In this case, the lack of activity might reasonably be ascribed to impermeability of the tissue, for Germanin has mol. wt. = *c.* 1500 and contains 6 sulphonic acid groups. Such an explanation however could not account for the non-activity of Trypan blue, which was observed to stain the brain slices deeply and uniformly.

The inertness of picric acid (used as Na salt) is worthy of note; for comparison one observation with dinitro-*o*-cresol is included in Table VIII, which confirms the effect of this compound on brain metabolism observed by Greville [1939]. It may be that the optimum concentration was not used, but in the experiment quoted the initial increase of respiration was the most striking effect and the clearness with which the Pasteur effect was inhibited lags far behind the experiment with guanidine and the same sample of tissue given in Table I. Dinitro-*o*-cresol shows some activity against relapsing fever spirochaetes [Fischl, 1934].

Diaminoacridine methochloride ("Gonacrine") is evidently one of the class of inhibitors of the Pasteur effect already described [Dickens, 1936], and is of similar activity to the rest of this group. On the other hand, Rivanol, containing

no quaternary N, has no appreciable effect on the Pasteur reaction, as appears to be the general rule with such compounds [Dickens, 1936].

Action of amidine compounds on carboxylase. Axmacher & Bergstermann [1934] showed that the action of yeast carboxylase on pyruvic acid was inhibited by Germanin. This effect, however, is not shown by substances containing the amidine group which are known to be trypanocidal (Table IX). Hence this action does not appear to be characteristic either of trypanocides or of inhibitors of the Pasteur effect.

Table IX. *Action on yeast carboxylase*

Each vessel contained in the main compartment 50 mg. carboxylase [Axmacher & Bergstermann, 1934] in 0.5 ml. *M*/20 phosphate buffer pH 6.2 together with 1.5 ml. 0.9% NaCl or 1.5 ml. *M*/200 substance in 0.9% NaCl. The side bulb held 0.2 ml. *M*/10 Na pyruvate. Final conc. of added substance *c.* *M*/300. Temp. 25°. Gas space, air. Decarboxylation was virtually complete in 30 min. in the control.

CO₂ evolved as % of control without added substance:

CO ₂ (Control = 100) after min.	Addition				
	Germanin	Hexadeca- methylen- diguandine	1:11-Undecan- diamidine	Deca- methylen- diisothiurea	Synthalin
5	66	99	102	98	101.5
10	79	97	97	95	98
15	85	97	95.5	94	97
20	86.5	97	95	93	96.5
30	89	100	93.5	92.5	96

DISCUSSION

Inhibition of the Pasteur effect. Like the action of cations, i.e. either excess K⁺ [Ashford & Dixon, 1935] or disturbance of physiological balance due to lack of K⁺ and Ca⁺⁺ [Dickens & Greville, 1935], or the presence of NH₄⁺ [Weil-Malherbe, 1938], that of the guanidine compounds described in this paper appears to be limited to, or most marked in, cerebral tissue. Possible reasons for this have already been discussed by Dickens & Greville [1935] and Dixon & Holmes [1935]. A further peculiarity of brain slices is that, owing to the anatomical structure of the brain, the preparation of the tissue slice must involve the cutting of numerous nerve-fibres and axons; hence in slices of cortical tissue, unlike those of the epithelial organs, almost every cell will present an injury-surface and this may cause an increased susceptibility to the composition of the suspension medium. While this may exaggerate an existing tendency it appears likely from the correspondence of *in vivo* effects with metabolic activities of the slice, that the *in vitro* actions of added substances such as those recorded here are not to be regarded as mere artefacts, but may well have real physiological significance. The present series of compounds is admirably suited for experimental investigation of their *in vivo* effects on brain metabolism, and it is hoped, in view of the often suggested connexion of guanidine compounds with tetany, that physiologists will examine the lactic acid content of brain tissue in guanidine and other forms of tetany.

As to the mechanism of action, no clear interpretation is possible at present. Two possible theories may be tentatively suggested, though neither is susceptible of proof. These are (1) depression of the ionization of Ca, (2) reaction of guanidine with tissue protein, resulting in its denaturation and probably in the liberation of SH groups. Apart from the inconclusive nature of the depression of ionization

or "inactivation" of Ca by guanidine derivatives [Frank *et al.* 1921; Thomson & Collip, 1932], in the present experiments the amount of guanidine derivative required is very much less than that of Ca^{++} present. The Ringer solution contained $M/500$ Ca^{++} , and only half this concentration of guanidine and 1/200th of this quantity of synthalin were necessary to inhibit completely the Pasteur effect in brain. It is, of course, possible that the inactivation of catalytically active amounts of Ca^{++} present at enzyme surfaces in the brain tissue could be caused even by these low concentrations. With regard to (2), it is well recognized that agents producing denaturation of protein are capable of causing irreversible inhibition of the Pasteur effect, which is in general the first metabolic sign of cell injury. Heat¹ [Kubowitz, 1929; Dickens *et al.* 1936; 1937; Dixon, 1936], or the use of Ringer solution instead of serum as a suspension medium for certain delicate tissues [Negelein, 1925] may cause inhibition of the Pasteur effect accompanied by a whitish appearance of tissue which probably indicates coagulation of protein. From a study of the published data it seems possible that the first metabolic effect of radium *in vivo* [Crabtree, 1932] or of X-rays *in vitro* [Bancroft & Kinsey, 1937] may have been irreversible injury to the Pasteur mechanism.

Such a destructive mechanism might act in several ways. It might bring about the direct destruction of some special "catalyst" of the Pasteur reaction such as Warburg [1926] supposed to exist; this compound would therefore be a highly thermolabile enzyme, since its irreversible inactivation proceeds at temperatures not exceeding 45° [Dickens *et al.* 1936; Dixon, 1936].

It has often been claimed that SH compounds may play an important part in regulating aerobic glycolysis [Quastel & Wheatley, 1932; Rapkine, 1931; 1938; Bumm & Appel, 1932; see however Baker, 1937; Weil-Malherbe, 1938]. Denaturation of proteins is frequently accompanied by liberation of SH groups, and it is possible that this property may be involved in the mechanism of the Pasteur reaction. One could imagine the "catalyst" of the Pasteur effect to be an enzyme having as its active group a protein -S-S linkage, which on denaturation would be transformed into the SH group. It is admittedly difficult to imagine that normally the mere transference of the tissue from aerobic to anaerobic conditions should be capable of causing such a change, which must, moreover, be reversible; Rapkine [1937] has however put forward a similar view of the changes in cellular division, which he supposes to be preceded by denaturation of protein. Without necessarily subscribing to these views, it may be pointed out that guanidine compounds are able to bring about denaturation of proteins [Greenstein, 1938; 1939] and their activity is considerably in excess of that of urea, amides etc. [Hopkins, 1930], as judged by the rate and extent of appearance of SH groups. The effect on the Pasteur reaction of the substances described in this paper presents a number of points of similarity with what might be expected from the resonance theory of Greenstein [1938]. For instance, methylguanidine and guanidine ions, which both show resonance and denature proteins, both inhibit the Pasteur effect; while the *as*-dimethylguanidonium ion does not appreciably inhibit the Pasteur effect, its resonance is greatly restricted, and it is a much less effective denaturing agent. (I have attempted to determine the denaturing ability towards ovalbumin of decamethylenediguandine and 1:11-undecane-

¹ Dixon [1936] considered that in brain tissue there was not a true inhibition of the Pasteur effect, but only an apparent one caused by the greater rate of fall of anaerobic than of aerobic glycolysis at 45°. However, in the experiments of Dickens *et al.* [1936] this rapid fall was often entirely absent, and Dixon's objection appears to us therefore not to apply to our observations, which were submitted for publication simultaneously with and independently of those of Dixon.

diamidine, the two most active inhibitors of the Pasteur reaction, but unfortunately their low solubility made this impossible except perhaps by the use of a very readily denatured protein.) The concentrations of guanidine compounds used by Greenstein were very much higher than those which inhibit the Pasteur effect, but one might expect the protein concerned in the latter to be more readily denatured than those employed by Greenstein. The most serious objection to this theory is the lack of any effect on metabolism of urea and acetamide, even in high concentrations; moreover, it is not known whether reversible denaturation can occur with guanidine compounds.

Specific antiseptics. The parallelism between antiseptic and metabolic activity, which up to a point is striking for chemically similar substances, breaks down when a wider variety of compounds are considered. Thus although certain quinoline and acridine antiseptics [Dickens, 1936], which are chemically quite unlike the guanidine compounds here described, show the same inhibition of the Pasteur effect in brain tissue, there are a number of powerful trypanocides which do not affect brain metabolism (e.g. Trypan blue, which stains the tissue deeply without affecting its respiration and glycolysis at all). It is not impossible, however, that the extension of these observations to the more complicated metabolism of the trypanosome might lead to results of value in the understanding of specific antiseptics: the most striking feature of the metabolism of *Trypanosoma Rhodesiense*, studied by Christophers & Fulton [1938], appears to be the large utilization of glucose accompanied by formation of acid. Since the respiration is known to be very sensitive to acid, an increased aerobic acid production might be of significance. However, the R.Q. of the trypanosome is only 0.2 and since the products formed from glucose are unknown there is little apparent resemblance between the metabolism of the parasite and of brain tissue.

Tetany. The similarity between the tetanic convulsions of guanidine poisoning and those following parathyroidectomy has led to numerous theories attributing tetany to intoxication with guanidine or its methyl derivatives; these are critically discussed by Thomson & Collip [1932].

It is by no means clear if the present observations have any direct connexion with tetany: it may however be remarked that in brain tissue the effects of absence of Ca^{++} from the medium [Dickens & Greville, 1935] or of addition of guanidine compounds are in the same direction, both causing increased respiration and aerobic glycolysis. On the other hand, dimethylguanidine, which has frequently been suggested as a cause of tetany, is much less active on the Pasteur effect than guanidine itself or its monomethyl derivatives.

Banu & Gavrilescu [1934] consider that the slight depression of brain respiration observed by them in the presence of high concentrations of guanidine may be, at least in part, the cause of guanidine tetany. It seems to the author that, if the effects described in this paper also occur *in vivo*, they might provide a far more probable explanation of guanidine tetany. The excessive aerobic acid production and unstable high metabolism observed in brain tissue, resembling in type that caused by lowered Ca^{++} content of the medium, would appear to be an action more likely to produce drastic pathological effects: it occurs moreover with concentrations of guanidine compounds which might not inconceivably be present in the animal organism in tetany.

SUMMARY

Addition to the medium of $M/1000$ guanidine completely and reversibly inhibits the Pasteur effect in slices of rat brain cortex, the respiration continuing usually at a higher level while the aerobic (lactic) acid formation attains or

exceeds the anaerobic. Complete restoration occurs on transference to guanidine-free medium. These effects are absent or less marked in other tissues and cells (amnion, medulla of kidney, Jensen sarcoma, baker's yeast). Lower concentrations ($5 \times 10^{-5} M$) are ineffective. Methylguanidine acts like guanidine, but the dimethyl derivative shows little typical action. Glycocyamine, arginine and creatine ($10^{-3} M$) are toxic; agmatine causes a slow inhibition of the Pasteur effect. Biuret ($10^{-3} M$) and $M/50$ urea and acetamide have no action on respiration or glycolysis.

Certain substituted guanidines and amidines are the most powerful inhibitors of the Pasteur effect yet described: these are 1:11-undecanediamidine and decamethylenediguanidine (Synthalin), which are fully active at 10^{-6} – $10^{-5} M$, the precise concentration depending on the relative amounts of solution and tissue. Both are powerful trypanocides; other specific antiseptics of the acridine and quinoline series have previously been found to inhibit the Pasteur effect in brain. This property is not however shared by all trypanocides, for Bayer 205 ($10^{-4} M$) and Trypan blue ($10^{-3} M$) do not affect brain metabolism. Decamethylenediisothiourea causes an incomplete inhibition of the Pasteur effect.

The bearing of these results on the mechanism of the Pasteur effect, and the possibility of their connexion with tetany and specific antiseptics are discussed.

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CCXLIX. THE ESTIMATION OF VITAMIN B₁ IN BLOOD

II. A FURTHER MODIFICATION OF MEIKLEJOHN'S METHOD

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MEIKLEJOHN [1937] described a method, based upon the work of Schopfer, of estimating vitamin B₁ in blood. The method uses the ability of the vitamin to promote the growth of a fungus, *Phycomyces blakesleeanus*. I have already shown that his claim "that the method . . . provides a quantitative estimate of the true vitamin B₁ content of the blood" is not justified because blood invariably contains substances other than vitamin B₁ that affect the growth of the fungus [Sinclair, 1938]. These substances fall into two groups. First, the method is not specific for vitamin B₁, since other substances can replace the vitamin as a growth factor. Such substances include the degradation products of the vitamin and certain compounds very closely related to these. They may be important since they can be produced by heating the vitamin in a slightly alkaline medium; for instance, the vitamin might be destroyed by cooking, but if a sample of blood was drawn shortly after the cooked food was eaten, the pyrimidine and thiazole components of the vitamin might be present in blood and so give a falsely high estimate by the test. Work using other fungi is in progress to test the importance of this possible error. In the tests that I have done during the last 3 years it has been avoided as far as possible by collecting most of the samples of blood at least 2 hr. after food was eaten. This paper is concerned with the second group of substances, namely those that have an adjuvant or inhibitory effect upon the growth of the fungus under the conditions of the test, but that cannot replace vitamin B₁ as a growth factor. I have shown that the adjuvant action of blood "is probably due to more than one factor; sources of nitrogen and salts in the blood, the buffering power of the blood and the more solid medium produced when blood is added, particularly in large amounts, probably all contribute to it. Under certain conditions, blood may be shown to have an inhibitory action on the growth of the fungus" [Sinclair, 1938]. One simple way of showing the presence of adjuvant substances was to add vitamin B₁ to blood: addition of small amounts of the vitamin usually produced a greater growth than expected and addition of excess vitamin invariably did so.

If one assumes that the growth produced by a given amount of vitamin B₁ in the presence of blood is always in the same ratio to the growth produced by the same amount of vitamin B₁ in the absence of blood, one can correct for the adjuvant action of blood by multiplying the weight of fungus obtained in the presence of blood by the factor:

$$\frac{\text{weight of fungus obtained with excess vitamin B}_1}{\text{weight of fungus obtained with excess vitamin B}_1 + \text{blood}}.$$

The crucial test of this assumption is to add known amounts of vitamin B₁ to blood from which the vitamin has been removed; but it has as yet proved

impossible to destroy the vitamin in blood without also affecting the adjuvant factor. It will be shown below that discrepancies obtained with Meiklejohn's method are eliminated when the correction is applied to allow for the adjuvant action of blood, using samples of blood that contain widely different amounts of vitamin B₁ (3–12 $\mu\text{g.}/100\text{ ml.}$), and adding various further amounts of the vitamin (0.025–0.2 $\mu\text{g.}$) to different volumes of blood (0.5–5.0 ml.). These results would appear to justify the assumption.

EXPERIMENTAL

The technique of the experiments has already been described [Sinclair, 1938]. In that paper I showed that samples of 1, 2 and 3 ml. blood usually did not give the same values per unit volume. Determinations upon samples of human blood from 235 different cases were then quoted. The number of relevant samples has now been increased to 425; the 3 ml. value (expressed in $\mu\text{g.}/100\text{ ml.}$ blood) has been at least 1 $\mu\text{g.}$ higher than the 1 ml. value in 61 %, at least 1 $\mu\text{g.}$ lower in 14 %; in 54 samples the growth with 3 ml. blood was greater than that given by excess vitamin. When the values are corrected, the different volumes of blood give approximately the same values per unit volume (Table I). In

Table I. *Comparison of corrected and uncorrected estimates of vitamin B₁ ($\mu\text{g.}/100\text{ ml.}$) obtained with different volumes of blood*

	Vol. of blood ml.	Sample of blood										
		1	2	3	4	5	6	7	8	9	10	11
Uncorrected	1	8	10	7.5	12.5	14.5	17	7.5	12	9	10.5	5
	2	9.5	11	8	—	20	13	16	14	10.5	—	7.5
	3	10.5	—	13.5	20.5	?	?	?	?	18	20	7.5
Corrected	1	7	7.5	3.5	11	7.5	12	7	8	6.5	9	4
	2	7	7	3	—	7.5	10	6.5	9	6.5	—	4.5
	3	8.5	—	3	10	7.5	12	7	8.5	8	10	4.5

* In some cases the growth of fungus was greater than that obtained with excess of added vitamin in absence of blood. In such cases it is not possible to translate the weights of growth into apparent amounts of vitamin B₁ and a question-mark has been placed in the table to indicate this.

Table I uncorrected values are compared with the corresponding corrected ones; the mean of the uncorrected 3 ml. values is 15 $\mu\text{g.}/100\text{ ml.}$ and the mean of the corresponding 1 ml. values is 8.8; the means of the 3 and 1 ml. corrected values are 7.9 and 7.6 respectively.

In my previous paper I showed that adding small amounts of vitamin B₁ to blood usually produced a greater growth than was expected. When the weights of fungus are corrected this discrepancy disappears. The results of six typical experiments are summarized in Table II, in which the figures in column *a* represent the apparent amount of vitamin B₁ in the blood (expressed in $\mu\text{g.}/100\text{ ml.}$ blood) estimated by subtracting the amount of added vitamin from the estimated total vitamin content; the figures in column *b* represent the corresponding corrected values. It will be seen that whereas the apparent values may vary widely, the corrected values agree within the limits of experimental error. The degree of variation is indicated by the standard deviation of the individual observations in each column. But in the case of the uncorrected values, it must be remembered that some growths of fungus were too large to be translated into apparent amounts of vitamin B₁; in these cases the estimate of the variation is therefore a minimal one.

Table II. *Effect of added vitamin B₁ upon the estimated vitamin content of different samples of blood (column a, uncorrected values; column b corrected values, $\mu\text{g.}/100\text{ ml.}$)*

Blood ml.	Added vitamin B ₁ $\mu\text{g.}$	Sample of blood											
		1		2		3		4		5		6	
		a	b	a	b	a	b	a	b	a	b	a	b
0.5	0	11	9	—	—	—	—	—	—	—	—	—	—
0.5	0.05	9	6	—	—	—	—	—	—	—	—	—	—
0.5	0.1	6	2	—	—	—	—	—	—	—	—	—	—
0.5	0.15	8.5	2	—	—	—	—	—	—	—	—	—	—
0.5	0.2	15	7	—	—	—	—	—	—	—	—	—	—
1.0	0	8	7	10	7.5	7.5	3.5	12.5	11	14.5	7.5	5	4
1.0	0.025	—	—	11	7.5	8	3	14.5	10.5	—	—	—	—
1.0	0.05	10	8	11.5	7	9	3	16	12	—	—	5.5	4
1.0	0.1	8.5	6	16	9	11.5	2	15	12	40	8	8.5	4
1.0	0.15	13	8.5	31	11.5	—	—	—	—	—	—	—	—
1.0	0.2	18	6	?	11	—	—	—	—	—	—	—	—
2.0	0	9.5	7	11	7	8	3	—	—	20	7.5	7.5	4.5
2.0	0.025	—	—	13	8	9	3	—	—	—	—	—	—
2.0	0.05	10.5	7.5	19	8.5	11	3	—	—	—	—	8	6
2.0	0.1	9	6	?	9.5	16	2	—	—	?	8	—	—
2.0	0.15	8.5	5.5	—	—	—	—	—	—	—	—	—	—
2.0	0.2	7	4	?	9	—	—	—	—	—	—	—	—
3.0	0	10.5	8.5	?	—	13.5	3	20.5	10	?	7.5	7.5	4.5
3.0	0.05	9.5	7.5	?	—	—	—	?	9.5	?	7.5	?	—
3.0	0.1	9.5	7	?	—	—	—	?	10	?	8	?	5.5
3.0	0.2	?	5.5	?	—	—	—	—	—	?	—	—	—
4.0	0	?	7.5	?	—	11.5	2.5	—	—	?	9	—	—
4.0	0.05	?	6.5	?	—	—	—	—	—	—	—	—	—
4.0	0.1	?	5.5	?	—	—	—	—	—	?	8	—	—
5.0	0	?	5.5	—	—	—	—	—	—	?	—	—	—
5.0	0.05	?	5.5	—	—	—	—	—	—	—	—	—	—
Mean		10.4	6.3	15.3	8.7	10.5	2.8	15.7	10.7	24.8	7.9	7.0	4.6
σ		2.8	1.8	7.0	1.6	2.7	0.5	3.0	0.9	13.4	0.5	1.4	0.8

A further experiment upon human blood is recorded in detail in Table III in order to illustrate the method of calculation and the variation between duplicate estimations. Again it will be seen that the corrected values agree extremely well considering the experimental error: for instance, the only bad duplicate estimations are the values of 6 and 12 $\mu\text{g.}/100\text{ ml.}$ obtained with 0.1 $\mu\text{g.}$ vitamin B₁ added to 0.5 ml. blood; but the difference in the corrected weights that produces this large difference in the values is only 6 mg.

A further fact is illustrated in Tables II and III. I have previously shown that "large samples (more than 3 ml.) of blood (to which no vitamin has been added) usually give a growth that is greater than that obtained with excess vitamin B₁" [Sinclair, 1938]. When the weight of this growth is corrected it invariably becomes less than that obtained with excess vitamin, and so the vitamin content of the blood can be estimated. Tables II and III contain examples of this. The largest value for vitamin B₁ in human blood that I have obtained in about 500 estimations was given by a case of myeloid leukaemia. The weights of fungus (mg.) were as follows:

Excess vitamin B ₁ (2.5 $\mu\text{g.}$)	90.8
1 ml. blood	122.7
1 ml. blood + excess vitamin B ₁	160.8
2 ml. blood	143.3
3 ml. blood	105.2

Blood ml.	Added vitamin B ₁ μg.	Wt. of fungus mg.	Vitamin equivalent μg.	Apparent vitamin content of blood μg./100 ml.	Corrected wt. of fungus mg.	Vitamin equivalent μg.	True vitamin content of blood μg./100 ml.
0	2.5	100.5 99.6	2.5	—	—	—	—
0.5	0	20.0 18.9	0.05 0.045	10 9	18.3 17.3	0.04 0.04	8 8
0.5	0.05	39.3 37.0	0.1 0.095	10 9	36.0 34.0	0.09 0.085	8 7
0.5	0.1	56.3 49.7	0.185 0.15	17 10	51.6 45.6	0.16 0.13	12 6
0.5	2.5	111.1 107.1	> 2.5	—	—	—	—
1.0	0	32.2 29.8	0.08 0.07	8 7	28.4 26.3	0.07 0.065	7 6.5
1.0	0.05	51.4 48.4	0.16 0.14	11 9	45.4 42.7	0.13 0.12	8 7
1.0	0.1	68.7 68.2	0.25 0.25	15 15	60.6 60.3	0.205 0.205	10.5 10.5
1.0	0.2	80.6 80.0	0.345 0.34	14.5 14	71.3 70.7	0.27 0.27	7 7
1.0	2.5	113.6 112.8	> 2.5	—	—	—	—
2.0	0	78.0 78.0	0.32 0.32	16 16	45.6 45.6	0.13 0.13	6.5 6.5
2.0	0.05	98.0 94.8	2.5	?	57.3 55.5	0.19 0.18	7 6.5
2.0	0.1	119.8 116.7	> 2.5	?	70.1 68.4	0.26 0.25	8 7.5
2.0	0.15	124.3	> 2.5	?	72.8	0.28	6.5
2.0	0.2	140.8	> 2.5	?	82.4	0.36	8
2.0	2.5	172.6 169.6	> 2.5	—	—	—	—
Mean				11.9	7.7		
σ				3.3	1.4		

Obviously Meiklejohn's method gives no estimate of the amount of vitamin in the blood, since without added vitamin the samples of blood gave greater growths than that obtained with excess vitamin alone. But the corrected weight for 1 ml. blood is $122.7 \times \frac{90.8}{160.8} = 69.4$ mg., and from the growth-vitamin curve for this experiment, this weight is equivalent to about 0.41 μ g. vitamin B₁. The true amount of total vitamin B₁ in the blood is therefore about 41 μ g./100 ml. Independently the cocarboxylase (vitamin B₁ diphosphate ester) in the sample was estimated by Dr Robert Goodhart; the amount was 42 μ g./100 ml. Since almost all the vitamin in the blood of cases of myeloid leukaemia is in the phosphorylated form [Goodhart & Sinclair, 1939, 2], these values—one obtained by a chemical and the other by a biological method—agree well.

These results support the claim that a correction can be applied to allow for the adjuvant action of blood, the correction being based, as described above, upon the increase in weight of fungus produced by blood in presence of excess vitamin B₁. I believe that if the precautions described elsewhere are borne in

mind [Sinclair, 1938], the results obtained by Meiklejohn's method can be corrected to give an approximately true estimate of the total vitamin B₁ in blood.

The volume of blood tested

To the clinical procedure of estimating the degree of saturation of the body with vitamin B₁ by assaying the vitamin in blood, certain other facts are relevant. Meiklejohn [1937] recommended that duplicate estimations of the vitamin should be done on 1, 2 and 3 ml. samples of blood, and on a 2 ml. sample to which 0.1 μ g. vitamin B₁ was added. This requires 16 ml. blood. I have criticized the adding of 0.1 μ g. vitamin B₁ to 2 ml. blood because the values obtained (about 0.3 μ g. with normal human blood) are too large to be read accurately from the growth-vitamin curve [Sinclair, 1938]. The 3 ml. sample should always be omitted; it gives values that tend to be higher (expressed per unit volume) than those given by the smaller samples and therefore if it is omitted in a particular case because 16 ml. blood cannot be obtained the estimate of the vitamin content of the blood will probably be falsely low when compared with average values obtained with 1, 2 and 3 ml. samples. Further, the medium that contains 3 ml. blood usually becomes solid after tyndallization at pH 6.5, and it is then possible that all the vitamin is not available for the fungus. Other things being equal, the most accurate estimate is obtained with amounts of blood producing the largest growths of fungus that fall on the steep part of the growth-vitamin curve. The corresponding amount of vitamin is about 0.1 μ g. The best method of translating weights of fungus into amounts of vitamin B₁ is to plot the results obtained with the control series of flasks on a logarithmic scale. If this is done it is found that the logarithm of the weight of fungus is directly proportional to the logarithm of the amount of vitamin B₁ present between 0.05 and 0.25 μ g. vitamin B₁; the mid-point of the straight line corresponds to 0.12 μ g. This happens to be approximately the mean apparent value for vitamin in 1 ml. normal human blood. Therefore the apparent values for normal blood can, other things being equal, be estimated with greatest accuracy in 1 ml. blood. In the case of the corrected values, the mean ($\pm \sigma$) for vitamin B₁ in normal human blood is $7.4 \pm 1.4 \mu\text{g./100 ml.}$ Apparent values of 7 or less and true values of 4.5 or less may be regarded as being subnormal (see below). These values are the critical ones in estimations that are designed to show whether a given sample of blood contains a normal or subnormal amount of the vitamin. In terms of 2 ml. blood, these values become 0.14 and 0.09 μ g. respectively, and these figures fall on the steep part of the curve. It seems, therefore, that the most satisfactory test, in estimations that are designed to show whether a sample of blood contains an abnormally small amount of vitamin, is obtained with 2 ml. samples, with and without excess vitamin B₁ added; for duplicate estimations this requires a minimum of 8 ml. blood. In addition, a useful check is provided by doing similar determinations upon 1 ml. blood, without addition of vitamin, with 0.1 μ g. vitamin and with excess. Since the true values (expressed per unit volume of blood) are approximately the same with 1, 2 and 3 ml. samples of blood, it is legitimate to compare average values obtained on different samples of blood, even if the average values are not obtained with samples of the same volume. This is not true of the uncorrected values; with these, average values obtained with samples of the same volume must be compared. In the estimations that I have done during the last 3 years, the 3 ml. sample has sometimes, and the 2 ml. sample occasionally, been omitted. I shall therefore quote in this paper and papers now in preparation only values obtained with 1 ml. samples of blood.

The amount of vitamin B₁ in normal human blood

Few figures have been published for the amount of vitamin B₁ in normal human blood. To illustrate a particular point, Meiklejohn [1937] included in his paper results obtained with different volumes of human blood from six selected cases. The values ranged from 6.5 to 14.0 $\mu\text{g.}/100\text{ ml.}$ These figures, of course, were not intended to indicate the amount of vitamin in normal human blood; the lowest figure was obtained with blood from a case of malignant cachexia [Meiklejohn, personal communication]. Laurent & Sinclair [1938] published the results of two analyses of the apparent vitamin B₁ in blood, made by a modification of Meiklejohn's method, upon a patient with peripheral neuritis associated with pyloric stenosis; they stated that "a value below 7 $\mu\text{g.}$ per 100 ml. is regarded as being abnormal, and, out of about 200 determinations made by one of us (H. M. S.) on normal and pathological samples of blood, the first (4 $\mu\text{g.}$ per 100 ml.) is the lowest value that has been encountered".

Later, Rowlands & Wilkinson [1938] published a paper on "The clinical significance and estimation of blood vitamin B₁." Their paper has been misinterpreted, since subsequent writers have concluded, for instance, that Rowlands & Wilkinson were led by Schopfer's discovery "to devise a technique for the estimation of the vitamin in human blood", whereas in fact they appear to have used Meiklejohn's method without modification. They stated: "We have employed Schopfer's technique and applied it to the estimation of the vitamin B₁ content of human blood; our method is very similar to that since described by Meiklejohn [1937], details of which were kindly made available to us by Prof. R. A. Peters. The test we used was as follows." There follows a description of Meiklejohn's method without modification or further acknowledgement, and a list of experimental evidence in support of the method nearly all of which was originally put forward by Meiklejohn (whose name is mentioned in connexion with one item only—tests on deficient pigeons). This paper by Rowlands & Wilkinson is not considered in any detail here because their results, as they subsequently admitted, are statistically worthless (*Brit. med. J.* 1938, 2, 1060, 1110), but it may be noticed that in one part of their paper they regarded a figure of 5.5 $\mu\text{g.}/100\text{ ml.}$ as "gross deficiency" and elsewhere a figure below this value was considered a "normal value".

Recently Lehmann & Nielsen [1939] have applied Meiklejohn's method without modification, but state that a detailed account and criticism of his method will appear in another paper. They found that the values for 36 normal men ranged from 7 to 13 $\mu\text{g.}/100\text{ ml.}$, and for 10 normal women from 8 to 12; the average for each group was 9 $\mu\text{g.}/100\text{ ml.}$, but no statistical analysis is given. Morell [1938], in a paper entitled "Ein neuer biologischer Vitamin B₁-Test", comments at length on the paper of Rowlands & Wilkinson and describes what is in fact Meiklejohn's test without any reference to him; nothing new is added by this paper. Guhr [1939] includes Meiklejohn's paper in his bibliography but does not refer to it in his text; when he states that the work of Schopfer & Jung has been extended by English investigations, he mentions only "Rowland und Wilkinson" and proceeds to describe what is in fact Meiklejohn's method. Guhr's results are, as he admits, surprising. The amount of vitamin B₁ in the blood of ten normal subjects varied from 8 to 10 $\mu\text{g.}/100\text{ ml.}$; but six normal hospital sisters, who had no signs or symptoms of deficiency of vitamin B₁, gave very low values: one gave 0 $\mu\text{g.}/100\text{ ml.}$ on one occasion and "traces" on another; four others gave "traces" and the sixth sister gave a value of 5 $\mu\text{g.}/100\text{ ml.}$ Five patients, none of whom might be expected to be deficient, gave values

between 3.8 and 10.8 $\mu\text{g./100 ml.}$ (the mean $\pm\sigma$ may be calculated to be 7.3 ± 2.6); 19 pregnant or puerperal women gave values that ranged from 0 (4 cases) or "traces" (6 cases) up to 18 $\mu\text{g./100 ml.}$ Guhr concludes that the physiological variation of vitamin B₁ in the blood can be very wide, and normal people may have no vitamin B₁ in their blood—a remarkable discovery.

The thiochrome method of Jansen has been applied to whole blood or plasma [Jansen, 1938]. Various modifications of the method have been introduced. According to Schneider & Burger [1938] the average amount of vitamin B₁ in normal serum is 6.4 $\mu\text{g./100 ml.}$; their values on 12 subjects ranged from 3.8 to 10.0; from their figures the mean ($\pm\sigma$) may be calculated to be 6.4 ± 1.9 . Widenbauer *et al.* [1939] obtained a mean value of 7 $\mu\text{g./100 ml.}$ on serum from 20 normal subjects of various ages; their values ranged from 2 to 11, but as the individual figures are not given σ cannot be calculated. Ritsert [1939] obtained a range of 3–15 $\mu\text{g./100 ml.}$ blood from 12 normal persons; the mean ($\pm\sigma$) may be calculated to be 7.9 ± 3.9 . Therefore from his figures the range within which 95 out of 100 observations might be expected to fall is 0.1–15.7 $\mu\text{g./100 ml.}$, or if the values are expressed to the nearest 0.5 $\mu\text{g.}$, a value of zero cannot be considered abnormally low; if the number of observations (12) is taken into consideration when interpreting the statistics, a value of zero is certainly not significantly low. Ritsert was unable to find cocarboxylase in blood. Hennessy & Cerecedo [1939] have published a modification of the thiochrome method that will estimate either free vitamin B₁ or cocarboxylase. They quote no figures for the latter in blood, but give the range 9–12 $\mu\text{g./100 ml.}$ for the former; the number of observations is not recorded. Benacchio [1939] obtained values of 3.6 and 3.2 $\mu\text{g./100 ml.}$ serum. Gachtgens [1939] has also published values obtained by the thiochrome method, but I have been unable to obtain his book.

Recently Heyns [1939] has described a yeast-test which is applicable to blood; no actual values for human blood are given, but two tests with this showed practically no total vitamin B₁. Goodhart & Sinclair [1939, 1, 2] have described a method for the estimation of cocarboxylase in blood, based upon the method of Ochoa & Peters [1938]. On 26 healthy adults they obtained a mean value ($\pm\sigma$) of $7.0 \pm 2.1 \mu\text{g./100 ml.}$ Because most of the vitamin in blood is phosphorylated and associated with the corpuscles the blood cell count is of importance in considering the amount of vitamin B₁ in blood [Sinclair, 1939, 1], and values for plasma or serum tend to be unreliable because they are greatly affected by slight haemolysis of the blood [Jansen, 1938]. By a combination of the method described by Goodhart & Sinclair and of the modification of Meiklejohn's method described in the present paper, both total vitamin B₁ and cocarboxylase may be estimated on 10 ml. blood; by these methods the metabolism of the vitamin has been partly elucidated [Sinclair, 1939, 2]. Some of the discrepancies between the results mentioned above are probably due to failure to recognize that most of the vitamin in blood is phosphorylated and bound to protein [Sinclair, 1939, 2].

Using the modification of Meiklejohn's method already described [Sinclair, 1938] (which estimates both free vitamin B₁ and cocarboxylase, whether bound or not) the following results have been obtained upon healthy adults during the last 3 years. Forty-four observations upon different samples of my own blood taken throughout this period and under various conditions (such as after fasting for 24 hr. or after being on a diet low in vitamin B₁ for a week) have given apparent values that range between 8.0 and 13.5, with a mean ($\pm\sigma$) of $9.5 \pm 1.5 \mu\text{g./100 ml.}$ In 15 cases the corresponding true values have been calculated: they range from 5.0 to 9.0 with a mean of $6.2 \pm 1.1 \mu\text{g./100 ml.}$ A

group of 73 "normal" adults has included 47 males and 26 females. They consisted of healthy medical students, members of the staff of this Department and of a local hospital, and surgical patients (for instance, cases of fractures). Their ages ranged from 17 to 78 years. A statistical analysis of the figures in the male and in the female groups has shown that there is no significant difference between the means of the two groups. Including all the 73 figures together, the apparent values for vitamin B₁ range from 8.0 to 15.0, with a mean of $10.6 \pm 1.7 \mu\text{g./100 ml.}$ Since 95 out of 100 observations may be expected to fall within the range of $\pm 2\sigma$, the range of apparent values in normal adults may be considered to be 7.2–14.0 $\mu\text{g./100 ml.}$, and a value of 7.0 or less can be considered to be abnormally low. The corresponding true values, obtained on 45 of the subjects, ranged from 5.5 to 10.5 with a mean of 7.4 ± 1.4 . The range of true values for vitamin B₁ in normal adults may therefore be considered to be 4.6–10.2 $\mu\text{g./100 ml.}$, and a value of 4.5 or less can be considered to be abnormally low. These results are shown graphically in Fig. 1. It is clear from this figure, and even more obvious

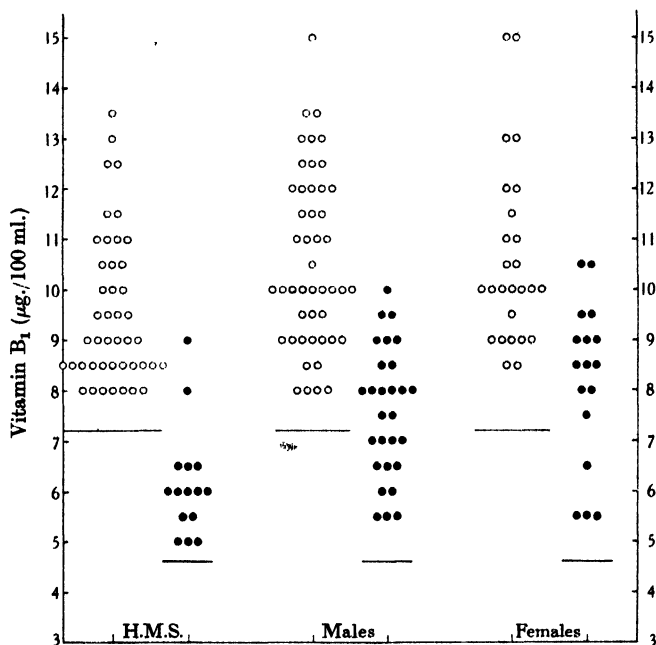


Fig. 1. Vitamin B₁ in the blood of healthy adults. (Circles, apparent values; dots, true values. The lines are drawn at the level mean -2σ calculated from the 73 apparent and 45 true values.)

if the frequency-distribution curve is plotted, that the individual observations are not normally distributed about the arithmetic mean. But in calculating the standard deviation on the assumption of a normal distribution, a greater scatter amongst the low values has been allowed for than in fact occurs, and therefore the lower limit, mean -2σ , becomes a more rigid test of the normality of an individual observation.

DISCUSSION

The uselessness of most of the work done so far upon the amount of vitamin B₁ in blood or serum is distressing. The arbitrary way in which Rowlands & Wilkinson [1938] call a figure of 5.5 $\mu\text{g./100 ml.}$ "gross deficiency" when a

figure below this is called a "normal value" has already been mentioned. Apart from workers from this Department, none of the authors concerned have treated their figures statistically, although some of them will doubtless do so when they report their results in greater detail. In the hands of Guhr [1939] Meiklejohn's method seems useless for clinical purposes because a value of 0 for total vitamin B₁ can be obtained with the blood of healthy adults. But I am encouraged to believe that my results are more trustworthy than Guhr's by the fact that Lehmann & Nielsen [1939] have obtained results very similar to mine. The latter authors, who used Meiklejohn's method, obtained a range of 7–14 $\mu\text{g.}/100\text{ ml.}$ upon 46 normal subjects; as already mentioned I obtained a range of 8–15 upon 73 normal subjects. I look forward to the promised publication of a critical analysis of Meiklejohn's method by these authors.

Turning to the thiochrome method, this promises to be useful for whole blood, and even for serum or plasma if great care is taken to avoid haemolysis or contamination with blood cells and if the vitamin is freed from protein by warming or digestion with pepsin. Although the results obtained by Ritser [1939] for unphosphorylated vitamin B₁ in blood are not encouraging since a value of zero is not significantly low, the modification of Hennessy & Cerecedo [1939] may prove valuable. It is, however, surprising that the latter authors obtained such high values for unphosphorylated vitamin B₁ in blood (9–12 $\mu\text{g.}/100\text{ ml.}$), and still more surprising that Ritser found no cocarboxylase in blood; in this Department we have found most of the vitamin in blood to be in the phosphorylated form [Goodhart & Sinclair, 1939, 2], and Heyns [1939] has also stated that by far the largest amount of vitamin B₁ occurs in blood in the form of cocarboxylase, although he is probably referring to the blood of animals other than man since his two tests with human blood yielded practically no result. It is also surprising that Schneider & Burger [1938] and Widenbauer *et al.* [1939] obtained average values of between 6 and 7 $\mu\text{g.}$ in 100 ml. serum, using the thiochrome method; Meiklejohn [1937], using a concentration of oxalate that may be haemolytic, found that about 20% of the vitamin B₁ in blood was present in plasma, and I obtained an average value of 1.1 $\mu\text{g.}/100\text{ ml.}$ (i.e. about 14% of the figure for blood) for normal plasma and serum, taking care to avoid haemolysis.

Considering all the above facts, it seems that the total vitamin B₁ in blood or in serum can be most accurately estimated by the modification of Meiklejohn's method that I have described in this paper, and that a value of 4.5 $\mu\text{g.}/100\text{ ml.}$ or less for human blood is significantly low.

SUMMARY

1. A modification of Meiklejohn's method for the estimation of total vitamin B₁ in blood is described, whereby a correction can be made for the adjuvant action of blood upon the growth of the fungus, and the apparent values obtained by the original method can be translated into true values. The method estimates both free vitamin B₁ and cocarboxylase.

2. Samples of 2 ml. blood are the most satisfactory, and the test can be performed in duplicate with a total of 8 ml.

3. Various workers have attempted to assay vitamin B₁ in blood or serum. Their results are mentioned and briefly criticized.

4. The true values for total vitamin B₁ in the blood of healthy adults of both sexes ranged from 5.5 to 10.5 $\mu\text{g.}/100\text{ ml.}$ with a mean ($\pm\sigma$) of 7.4 ± 1.4 . A value of 4.5 or less can be considered to be abnormally low.

I am very grateful to Prof. R. A. Peters for his interest and advice.

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CCL. ASSESSMENT OF THE LEVEL OF NUTRITION. A METHOD FOR THE ESTIMATION OF NICOTINIC ACID IN URINE¹

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WHEN in 1937 the pellagra-preventing factor was identified as nicotinic acid it became desirable to try and devise a method to estimate it in urine, with the object of working out a test for determining the "level of nutrition" similar to that already used for vitamins C [Harris & Ray, 1935] and B₁ [Harris & Leong, 1936].

After consultation with Dr S. Greenburgh, Public Analyst at Cambridge, it was decided to investigate the possibilities of three alternative methods of estimation.

Methods for estimating nicotinic acid

(1) The first of these to be considered, decarboxylation of nicotinic acid yielding pyridine and recovery of the latter after distillation, was judged to be unsatisfactory and inconvenient as compared with the procedure finally adopted.

(2) A second method, namely a colour reaction given by pyridine derivatives with 2:4-dinitrochlorobenzene [Vongerichten, 1899; Zincke, 1904], was also examined but was abandoned after a time as we reached the conclusion that it was more suited for a rough qualitative test than for a specific quantitative determination. This reaction has already been recommended by Vilter *et al.* [1938] for estimating nicotinic acid in urine, but it must be mentioned that the values which they cite as obtained by its use are widely divergent from those to be recorded in this paper.

(3) The method which we finally chose was based on the colour test for pyridine derivatives introduced by König in 1904, namely the reaction with cyanogen bromide plus an aromatic amine. A variation of this reaction was used by Strafford & Parry Jones [1933] and by Barta [1935] for detecting pyridine present as an impurity in nicotine. Swaminathan [1938] and Bandier & Hald [1939] and others have recently described a test for nicotinic acid based upon this reaction, but in the form set out by these workers the test does not, as we find, actually permit of the quantitative determination of nicotinic acid in urine. As Euler *et al.* [1938] have pointed out, the depth of the coloration given by pyridine derivatives in a König test depends on the pH, on the concentration of salts, and on other variable factors. By the procedure described in the present paper we have however been able to circumvent these disturbing influences.

¹ Communicated to the Biochemical Society 1 July 1939 [Harris & Raymond, 1939].

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Chemistry of the König reaction

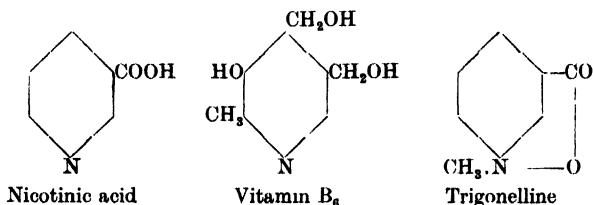
According to König [1904] the general type of the interaction of pyridine derivatives with CNBr and aromatic amines follows a course of which the special features are the formation of an addition compound with CNBr, the substitution of two or more aromatic nuclei into the resulting compound, followed by the possible migration of one H atom and accompanied by an opening of the ring structure. König considered that with increasing concentrations of the amine an increasing number of aromatic groups might be forced into combination.

EXPERIMENTAL

The work now to be described falls roughly under three headings: first a series of preliminary control tests to examine the specificity of the reaction; secondly a study of the more exact quantitative relations of the reaction, with the object of devising an accurate analytical procedure; and thirdly, the application of this method to controlled biological material.

Preliminary control tests

(a) *Specificity.* Since it has recently been shown that vitamin B₆ is, like nicotinic acid, a pyridine derivative it was thought advisable to examine its behaviour in the colour reaction. Fortunately it was found not to interfere under



the conditions of our test, carried out as specified below. In confirmation of the observation of Swaminathan [1938] trigonelline, a closely related compound which may also occur in urine, was likewise found not to yield a König reaction.

(b) *Varying intensity of colour.* Preliminary experiments at once made it obvious that the colour developed was very sensitive and changed in intensity as the pH was altered, or as salts were added to the medium, and with other variables, as found by Euler *et al.* [1938]. Later work, described in the quantitative section, was therefore designed to try and overcome these difficulties.

(c) *Necessity for blank.* Substances presumably of an aldehydic nature were found to be present in urine and to give a colour with the aromatic amine even in absence of CNBr. Accordingly it was concluded that a "blank" test without CNBr must form part of the procedure in order to allow for such "non-specific" coloration.

(d) *Choice of aromatic amine.* A large number of miscellaneous aromatic bases, including those listed in Table I, were examined in turn with the object of discovering which seemed most suitable for the test. It will be noticed that among the substances tried was *p*-aminoacetophenone which was thought of because of its previous use by Prebluda & McCollum [1937] as a colour reagent for vitamin B₁. This substance was finally chosen as having the following advantages among others:

(1) The colour is relatively stable if kept protected from light.

(2) The "blank" for the reagents, i.e. all reagents, mixed together in absence only of nicotinic acid, is quite colourless.

Table I. *Aromatic amines tested*

Metol
 Aniline
 Benzidine
o-, *m*- and *p*-Toluidine
 α - and β -Naphthylamine
 Dianisidine
 α -Aminoanthroquinone
 2:4-Dichloraniline
p-Dimethylaminoaniline
 1-Amino-2-naphthol-4-sulphonic acid
p-Aminoacetophenone

(3) The intensity of the colour is unusually high, e.g. about five times that given by metol or aniline.

(4) The colour can be extracted by ethyl acetate, and this property may prove, in certain modifications of the test, suitable for use for other biological assays.

Aniline, used by Swaminathan, and metol used by Bandier & Hald seemed less satisfactory in our hands for these and other reasons.

Quantitative relationships

In the more detailed quantitative investigation of the reaction, undertaken with the object of elaborating a reliable analytical procedure, attention had to be paid to all of the following seven factors: (a) influence of light, (b) influence of pH and salts, (c) effect of variations in concentration of the amine reagent used, (d) effect of variations in concentration of CNBr, (e) action of excess acid, (f) applicability of reaction to nicotinamide, (g) limits of sensitivity.

Unless otherwise stated values for colour intensity given below were those obtained by the standardized procedure set out later in this paper.

(a) *Effect of exposure to light.* Early in the work it was found that the fully developed colour, given at the last stage of the reaction, faded fairly quickly on exposure to light. Control tests (e.g. Table II) indicated that, provided this

Table II. *Effect of light*

Time min.	<i>E</i>	
	Solution kept in dark	Solution exposed to light
0	1.47	1.47
15	1.47	1.2
30	1.47	<1.2
60	1.47	—

coloured solution were kept in the dark, no appreciable fading occurred over a reasonably long time. For this reason we have stipulated in the working directions that certain of the operations must be carried out in such a way as to prevent exposure to bright light.

We are indebted to Dr E. Kodicek for the observation that when more dilute solutions are employed than that referred to in Table II, some measurable fading will occur within a period of say about 1 hr. even in the dark. Nevertheless, under the actual working conditions of our test, the loss in an experimental period of about 15–30 min. is still so small as to be of no practical significance, provided that the solution is kept in the dark.

(b) *Change of pH by buffer.* The striking effect of the addition of a buffer mixture on the intensity of the colour is sufficiently illustrated by the one example given in Fig. 1. It will be noticed that under the particular conditions of the experiment in question the presence of an acetate buffer at pH c. 3 sufficed to reduce the colour to about one-third of its intensity for any particular concentration of nicotinic acid. But it is important to note that for any given set of conditions, either for the pure unbuffered solution of nicotinic acid (upper curve) or for the solution with the addition of a fixed amount of buffer (lower curve), the relation between colour intensity and concentration of the unknown (nicotinic acid) remains a linear one—i.e. the system within these limits obeys Beer's law. Use is made of this fact in the method of test to be described.

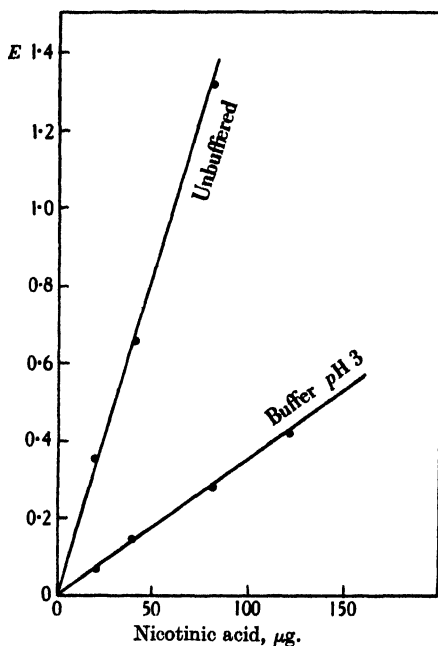


Fig. 1.

Fig. 1. Influence of buffer on slope of curve. Above, unbuffered solution. Below, in presence of acetate buffer at pH c. 3.

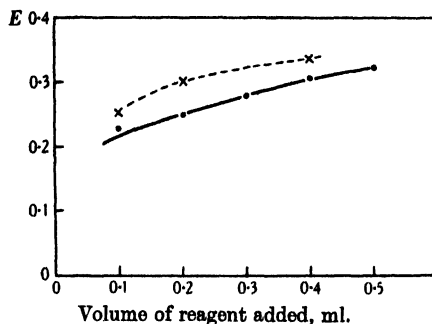


Fig. 2.

Fig. 2. Effect of varying the amount of aromatic amine added. Other conditions, e.g. concentration of nicotinic acid, kept constant. • = results with aqueous solutions. x = results with urine.

(c) *Effect of variation in concentration of amine reagent.* As will be seen from Fig. 2 the intensity of the coloration increases only relatively slowly as the concentration of the amine reagent is increased, provided that the latter exceeds values of about 0.1 or 0.2 ml. While therefore it is important that in carrying out an estimation the amount of reagent added should be kept reasonably constant on all occasions, nevertheless any small variation in volume or concentration due to unavoidable experimental error will have no serious effect on the accuracy of the result.

(d) *Influence of variations in amount of CNBr used.* Again with the CNBr, the intensity of colour is increased as its concentration rises. However, provided that a reasonable excess is used, the effect is not significant.

(e) *Effect of acid.* Table III illustrates how, under a given set of circumstances, the addition of acid causes the intensity of colour gradually to rise and then as more acid is added it once more begins to diminish. It is essential therefore that the amount of acid should be kept constant for any particular test.

Table III. *Illustrative experiment showing effect of addition of acid on intensity of colour produced in a buffered solution of nicotinic acid*

Vol. of conc. HCl added to 1 vol. of urine	<i>E</i>
0.0	0.182
0.1	0.234
0.2	0.202
0.4	0.186

(f) *Conversion of nicotinamide into nicotinic acid.* As the pellagra-preventing factor in urine occurs partly as free nicotinic acid and partly as nicotinamide, the procedure used must be capable of estimating both forms. The results given in Table IV indicate that the preliminary hydrolysis which we employ is adequate to convert the amide into the acid and give quantitative recovery. The question of the possible occurrence and estimation in urine of other related substances, e.g. nicotinuric acid [Ackermann, 1912], will be discussed in a later paper.

Table IV. *Recovery of added nicotinic acid*

Description	Amount taken $\mu\text{g.}$	Amount found $\mu\text{g.}$	Recovery %
Aqueous solution	98	103	105
	39.3	41	104
Added to urine	98	96	98

(g) *Limit of sensitivity.* With the use of the standard procedure described below, it has been found possible to detect as little as 1 $\mu\text{g.}$ of nicotinic acid. Below this amount, detection would be difficult.

PRINCIPLE OF METHOD RECOMMENDED

The special feature of the method to be described is that the urine, after preliminary heating with NaOH to convert any amide into the acid, is divided into four equal portions: one is kept as blank and to the other three nicotinic acid is added in known graded amounts, viz. 0, 20 and 40 $\mu\text{g.}$ These three solutions, which are kept protected from the light, are all treated in the same way, being in turn warmed with CNBr, cooled, treated with *p*-aminoacetophenone allowed to stand, acidified, and then their colour-intensities measured in a Pulfrich step photometer. From what has already been said in the preceding sections, it will be clear that for any given specimen the depth of colour will vary with pH and the concentration of salts (and with other factors); but these three readings are obtained under conditions which are identical except for the concentration of nicotinic acid added, and it is found therefore that if we plot the colour intensities (as ordinates) against the amounts of nicotinic acid added (as abscissae) the readings will always lie on a straight line. If this line is now produced backwards it will cut the X-axis at a distance from the origin which represents the amount of nicotinic acid which would have to be removed from the urine to give a zero content, that is the nicotinic acid content of the original urine itself.

Working details

Since success in the use of the method depends on close attention to details, it is advisable to describe the procedure in full.

Reagents. (1) *CNBr solution.* Prepared fresh daily by adding a 10% aqueous solution of KCN drop by drop to saturated Br water until it is just decolorized. (2) *Amine reagent.* *p*-Aminoacetophenone 5 g., plus HCl (10 g. HCl per 100 ml.) 14 ml., plus distilled water to 50 ml.

Apparatus. Pulfrich step photometer, 15 ml. graduated stoppered flasks, pipettes, burettes, water bath with false bottom.

Process for urine. (1) *Hydrolysis of amide and esters of nicotinic acid.* A 25 ml. specimen of the urine is measured out, 5 ml. of 20% NaOH are added and the mixture heated for 30 min. on a steam bath to convert any nicotinamide into free nicotinic acid. The solution is next accurately neutralized, for which purpose 2 ml. of 4% NaHCO₃ is first added to help stabilize the end-point; and then concentrated HCl (usually about 1.8 ml.) is run in very cautiously drop by drop from a microburette reading to 0.01 ml. until the solution is precisely at pH 6 (bromothymol blue as external indicator). The contents of the flask and washings are then transferred to a 50 ml. graduated flask and made up to the mark.

Notes. (a) Excess of acid or alkali should be avoided as it adversely affects subsequent reactions.

(b) With urines rich in nicotinic acid dilution to 100, 200 or even 500 ml. may be necessary.

(2) *Reaction with CNBr and aromatic amine.* Four 15 ml. standard flasks are taken, labelled X, A, B and C. (X is the blank, A the unknown and B and C controls to which known amounts of nicotinic acid are added.) In B is placed 0.2 ml. and in C 0.4 ml. of a standard solution of nicotinic acid (100 μ g. = 1 ml.). By means of a pipette 10 ml. of the prepared urine are run into each of the flasks A, B, C and X, care being taken to wash down any of the strong solution of standard nicotinic acid adhering to the sides of the flask. All flasks are placed in an opaque-sided water bath and kept at 80° for c. 10 min. or until they acquire the temperature of the bath. CNBr reagent, 2 ml., is added to each of A, B and C but not X, the contents of the flasks being mixed by rotating them. At the end of a further 4 min. the flasks are removed to a bath of cold water (not exposed to strong light). After 4 min. cooling, 0.2 ml. of the aromatic amine reagent (solution no. 2) is added to each of the flasks A, B, C and X. The contents are mixed and the flasks are placed in a dark cupboard for 15 min.

(3) *Development of latent colour.* To each of flasks X, A, B and C 0.4 ml. of a 10% solution of HCl is added, the contents are diluted to the mark with distilled water and well mixed, and the flasks replaced in the dark cupboard. Approximately 15 min. after the addition of the acid, colorimetric measurements are made with the Pulfrich photometer. Solution X is placed in one 3 cm. cell and A, B and C introduced successively in the other 3 cm. cell, an S 47 filter being used. An exact match with solution C is sometimes difficult, on account of slight differences in the shade of the blue observed.

Notes. (a) It is important that all the flasks be similarly treated with respect to the addition of acid. Similarity of treatment is more important than the exact quantity or strength of the acid. A 2 ml. micro-burette divided into 0.01 ml. divisions is satisfactory for measuring the acid.

(b) Although it is not necessary to work in absolute darkness, the colours are extremely sensitive to light, especially so when in acid reaction. The solutions should be exposed as little as possible to strong daylight. The colours are sufficiently stable to allow accurate measurement in the photometer. The stages from the cooling onwards are best carried out in a darkened room.

(c) The CNBr is poisonous and should be added from a burette.

(d) Any turbidity in the treated urine may be a source of serious error. No difficulty is usually experienced with human urine, but urine from experimental animals often requires to be centrifuged before hydrolysis and recentrifuged after hydrolysis and then diluted to a known volume. If there is any marked difference in turbidity in the final coloured solutions X and A a third centrifuging may be necessary before the colour measurement. A preferable procedure [Kodicek, 1939] is to clarify by addition of 60 ml. of ethanol after the preliminary hydrolysis; this addition does not change the intensity or stability of the colour, or the recovery of nicotinic acid.

(e) *Preservation of urine.* Toluene used as preservative must be repurified ("sulphur-free"); otherwise the impurities present in it will interfere in the colour reaction. Urine preserved with such purified toluene retains the nicotinic acid well, for some days at least.

Calculations. From the three photometer readings (say k_A , k_B , k_C) a graph is constructed, taking these values as ordinates and the corresponding known amounts of nicotinic acid added (0, 20 and 40 $\mu\text{g.}$) as abscissae. A straight line is so obtained, which when produced backwards cuts the axis at a distance from the origin representing the amount of nicotinic acid in the specimen examined.

For routine work it is sufficient to prepare three aliquots (A, B and X) instead of four (A, B, C and X) and to calculate the result thus:

Nicotinic acid in $\mu\text{g. per ml.} = \frac{k_A}{(k_B - k_A)} \times \frac{20}{n}$ where n equals the number of ml. of urine taken in one aliquot of the diluted urine.

Examples illustrating principle of method. In Table V are entered the records of an experiment in which three separate estimates were made on one and the same specimen of urine, but under three different sets of experimental conditions. The data are treated graphically in Fig. 3, and it will be noted that

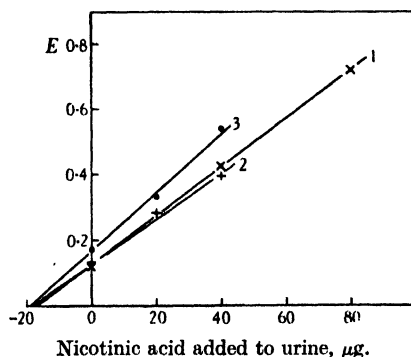


Fig. 3. Triplicate determinations on same specimen of urine under varying conditions, showing the agreement in the final results:

Curve 1, 40 and 80 $\mu\text{g.}$ added:	N.A. found = 17 $\mu\text{g.}$
2, 20 and 40 $\mu\text{g.}$ added:	N.A. found = 18 $\mu\text{g.}$
3, 20 and 40 $\mu\text{g.}$ added, less acid:	N.A. found = 19 $\mu\text{g.}$

notwithstanding the experimental variations in question, e.g. in the $p\text{H}$ or in the amounts of nicotinic acid added to the control tubes, the results obtained on the three occasions were yet in fairly good agreement, viz. 17, 18 and 19 $\mu\text{g.}$ of nicotinic acid in the 5 ml. of urine taken for examination. This example has been specially chosen to illustrate the underlying principle of the method, viz. that although the slope of the graph and the values of the initial readings on the urine itself may vary in determinations done under different conditions, nevertheless the results as obtained by extrapolation are always in good accord.

Table V. *Determination of nicotinic acid in urine under varying experimental conditions*

Nicotinic acid added, μg .	Exp. 1 (Determination no. 1) Photometer reading	Exp. 2 (Duplicate under somewhat different conditions) Photometer reading	Exp. 3 (Triplicate, with 0.1 ml. HCl instead of 0.2 ml.) Photometer reading
0	0.12	0.13	0.18
20	—	0.28	0.33
40	0.43	0.39	0.54
80	0.72	—	—
Nicotinic acid found, μg .	17	18	19

A second example is given in Table VI; and Fig. 4 illustrates how, when the corresponding values are plotted out, the same final result is reached from either of the two duplicate determinations.

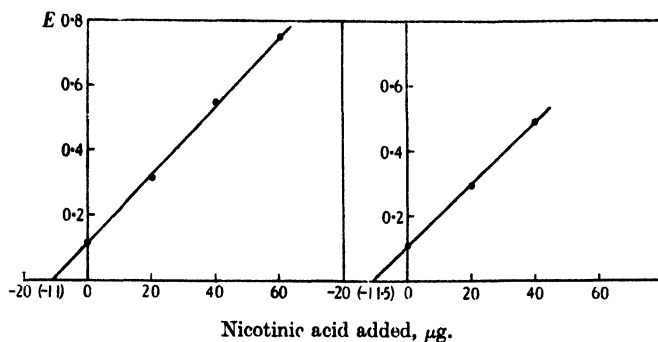


Fig. 4. Duplicate determinations, case of anorexia nervosa.

Value found, left = 11 μg .Value found, right = 11.5 μg .Table VI. *Duplicate determinations of nicotinic acid on single specimen of urine: case of anorexia nervosa*

Amount of nicotinic acid added, μg .	Photometer readings	
	First experiment	Second experiment
0	0.12	0.11
20	0.31	0.29
40	0.55	0.48
Nicotinic acid found	11 μg .	11.5 μg .

Degree of accuracy attainable. The figures given in the above tables for the photometer readings always represent the average of three readings. They are to be regarded as accurate to about ± 0.01 – 0.02 unit, and the points plotted from them are generally found to be on the straight line, within this same limit. From the results already cited, and from other determinations, it can be stated that duplicates agree well, within an experimental error of about $\pm 10\%$, and that added nicotinic acid or amide is quantitatively recovered.

TESTS ON NORMAL AND DEFICIENT EXPERIMENTAL ANIMALS

Since cases of pellagra are not easily available in this country we decided that control tests should be begun in the first place with experimental animals kept on various levels of adequacy or deficiency of nicotinic acid.

(a) *Experiments on guinea-pigs*

Gradual loss of nicotinic acid from urine during development of deficiency disease. As has been recorded elsewhere [Harris, 1939], guinea-pigs are unable to survive on a diet devoid of nicotinic acid. In the representative experiment illustrated in Fig. 5, one guinea-pig was kept on a regimen deficient in nicotinic acid (a modified Goldberger diet) while a second guinea-pig, serving as positive control, received daily supplements of the substance. The animal having no nicotinic acid began to show the first evidence of deficiency with a corresponding loss of weight after about 3 weeks, and his condition progressively deteriorated thereafter until he finally succumbed after 4-5 weeks; the positive control on the other hand continued to thrive. It will be noticed that as the deficiency disease advanced the amount of nicotinic acid in the urine fell steadily until at the last stages it had become virtually zero. It is important to recognize however that at the onset of the disease nicotinic acid was still being excreted in significant amounts, and that it was not until the animal was already severely ill that the excretion entirely ceased.

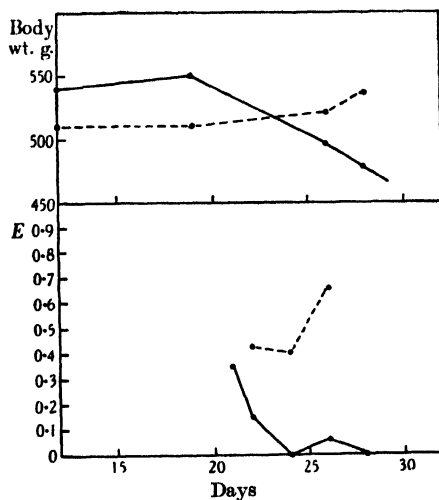


Fig. 5. Loss of nicotinic acid from urine of guinea-pig during development of deficiency. Above, weight curves. Below, colour value of urines.

— Guinea-pig on Goldberger diet.
 - - - Guinea-pig on Goldberger diet + nicotinic acid.

Excretion at various levels of intake. In continuation of these observations, groups of guinea-pigs were dosed with varying amounts of nicotinic acid. The graphs corresponding with two typical urinary analyses are illustrated in Figs. 6 and 7, the first indicating that there was a zero excretion by two guinea-pigs on a deficient diet and the second that there was an excretion of 400 μ g. daily by the two guinea-pigs receiving 2 mg. each daily. These charts are included

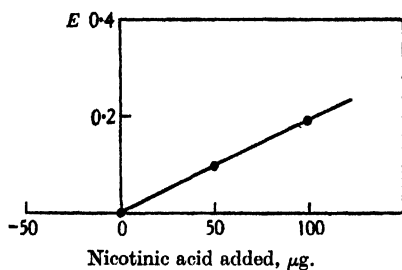


Fig. 6.

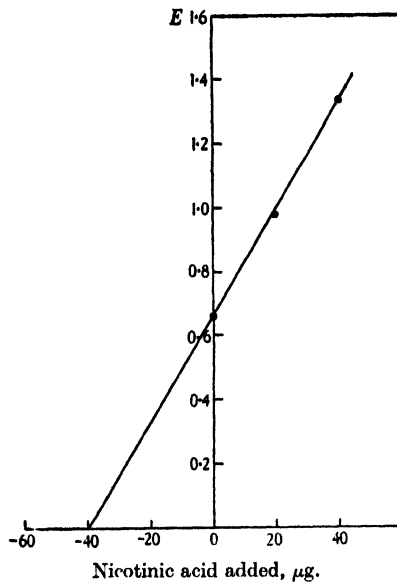


Fig. 7.

Fig. 6. Test on urine from two deficient guinea-pigs showing the absence of nicotinic acid (cf. Fig. 7).

Fig. 7. Test on urine of normal guinea-pig receiving 2 mg. nicotinic acid daily. Nicotinic acid found = 40 $\mu\text{g.}$ ($\equiv 400 \mu\text{g.}$ in 24 hr. specimen).

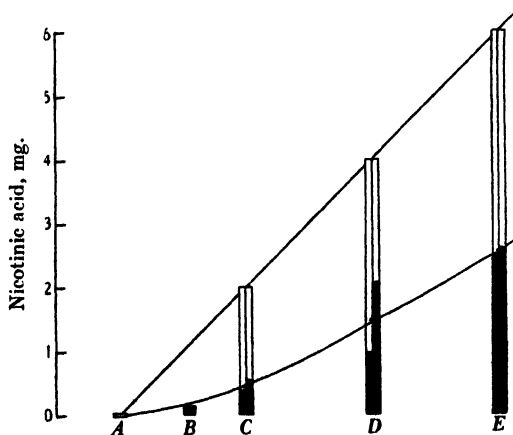


Fig. 8. Excretion of nicotinic acid with increasing level of intake.

Open columns = intake; black columns = excretion.

A = deficient diet.

B = stock diet of bran and oats, etc.

C = deficient diet + 2 mg. nicotinic acid.

D = deficient diet + 4 mg. nicotinic acid.

E = deficient diet + 6 mg. nicotinic acid.

The proportion excreted rises with increasing intake.

because they show that the method of calculation used with human urine is equally applicable for work with guinea-pigs (as we have found it also to be for dogs and other species); i.e. the experimental points always lie on a straight line, so that the amount of nicotinic acid originally present can be determined by extrapolation. The results of a series of such experiments are collected in Table VII. It is apparent that the percentage of nicotinic acid excreted rises continuously as the intake increases. The relation is more clearly seen from the graphical summary of results in Fig. 8.

Table VII. *Excretion of nicotinic acid by guinea-pigs*

Diet	mg.		% excreted
	Individual animals	Average	
Bran, oats, etc.	0.14; 0.14	0.14	—
Deficient	0.0; 0.0	0.0	0
Deficient + 2 mg. nicotinic acid	0.38; 0.54	0.46	23
Deficient + 4 mg. nicotinic acid	0.96; 2.06	1.51	38
Deficient + 6 mg. nicotinic acid	2.5; 2.6	2.55	44

(b) *Experiments on dogs*

Tests leading to similar results were also carried out with dogs. The animals were kept on a Goldberger blacktongue diet, with or without addition of yeast, liver extract (or other source of nicotinic acid) or nicotinic acid itself. Those dogs having no supplement duly developed blacktongue, and the amount of nicotinic acid in the urine was found to fall gradually as the disease progressed. Controls receiving the nicotinic acid, or the various addenda containing it, excreted nicotinic acid in corresponding amounts. These confirmatory experiments were carried out in collaboration with Dr E. Kodicek to whom we are indebted for the analyses in Table VIII.

Table VIII. *Gradual fall in excretion of nicotinic acid by dogs on diets deficient in P.P. factor*

Dog no.	Day of exp.	Diet	Nicotinic acid excreted $\mu\text{g. per day}$	Notes
1	5	Deficient	335	Goldberger diet (plus ribo-flavin and vitamin B ₁) to 30th day
	11	Deficient	250	
	18	Deficient	73	
	28	Deficient	0	Eli Lilly "343" liver powder, 4 g. daily, given from 30th to 45th day
	33	Supplemented with liver extract	108	
	45	Supplemented with liver extract	420	
	57	Deficient	0	Supplement discontinued from 45th day to end
2	5	Deficient	155	Goldberger diet to 30th day
	28	Deficient	0	
	33	Supplemented with nicotinic acid	336	Nicotinic acid, 10 mg. daily, given from 30th to 36th day
	45	Deficient	120	
	57	Deficient	0	
3	11	Positive control	347	Goldberger diet supplemented with Eli Lilly "343" liver powder, 4 g. daily
	18	Positive control	660	
4	28	Positive control	820	Goldberger diet supplemented with nicotinic acid, 2 mg. per kg. body weight daily

(c) Excretion of nicotinic acid by rats

Rats differ from the species hitherto discussed—man, guinea-pig and dog—in that they are able to remain free from symptoms of deficiency disease when kept on a Goldberger diet [Birch *et al.* 1935]. It was of interest therefore to examine the urine of such rats. We found that appreciable amounts of nicotinic acid were still excreted in the urine, even after the rats had been kept for long periods on the deficient diet (Table IX). The most likely explanation seems to be that the rat can synthesize nicotinic acid under certain conditions, either in its tissues or by a symbiotic process analogous with refection. This would account for the relative non-susceptibility of the rat as contrasted for example with man, monkey, pig or guinea-pig. The alternative view is that rats do need nicotinic acid but that a comparatively minute amount suffices in comparison with the larger requirements of other species. These problems can best be settled by balance experiments on rats kept on “synthetic” as well as on maize diets, an account of which will be published separately. In the meantime it may be noted that with large additions of nicotinic acid to the diet the excretion is further raised (Table IX).

Table IX. *Excretion of nicotinic acid by rats*

Diet	Daily excretion in urine (μ g.)
Basal diet	25, 25, 23, 12.5
Basal diet + nicotinic acid (10 mg. test dose)	100

Preliminary surveys on human subjects

Effect of test doses. Since the methods previously introduced for assessing the level of nutrition for vitamins C [Harris & Ray, 1935] and B₁ [Harris & Leong, 1936] involved the administration of test doses, to determine the “degree of

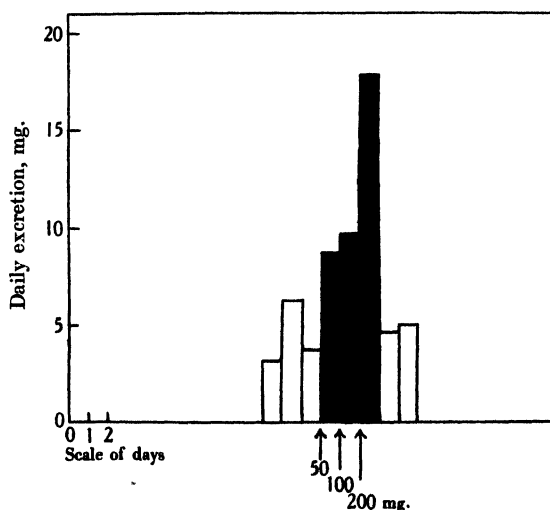


Fig. 9. Increased excretion of nicotinic acid by human subject after test doses (amounts given as shown at arrow).

saturation” of the tissues, it was decided to try out a similar procedure for nicotinic acid. From the tests on experimental animals already recorded it is

clear that with other species at any rate the excretion does in fact increase with the intake. A number of experiments upon ourselves, one of which is shown graphically in Fig. 9, indicate that a similar relation holds for human beings.

Range of resting values and dependence upon intake. Determinations on a group of presumed normal subjects (research workers and laboratory assistants) showed that the usual rate of excretion of nicotinic acid in the urine as determined by this method was about 4-5 mg. per day, the minimum value noted being 3.1 and the maximum 6.2; higher values were however seen when the diet had been enriched with foods especially active in the P.P. factor, e.g. marmite. These values, together with the results of test-dosing, are all collected together for easy reference in Table X.

Table X. *Excretion of nicotinic acid by human subjects, mg. per day*

Description	Resting levels	After test doses, mg.		
		50	100	200
Normal:				
L. J. H.	3.1, 6.2, 3.6, 4.6, 5.0	8.6	9.6	17.9
W. D. R.	3.5, 5.0	14.1	12.9	14.9
W. D. R. smoking heavily	7.3	---	---	---
W. D. R. smoking heavily + marmite	9.8, 12.6, 10.0	---	---	---
Y. L. W.	3.9	---	---	---
G. G. G.	4.9	---	---	---
Deficient:				
Anorexia nervosa	2.5, 2	---	---	---
Pellagra	1.8, 2.9	---	---	---
Pellagra during treatment	4.6, 4.8, 10	---	---	---
? Pellagra spinal-cord condition	2.76	---	---	---

An indication of increased values in subjects who are smoking heavily is worthy of further study.

Low values in disease. We have not yet examined any significant number of deficient subjects but, for the sake of comparison, included in Table X are references to three miscellaneous cases which we were able to investigate through the kindness of Dr Yudkin, Prof. Drummond and Dr Ungley, respectively. It is noteworthy that in these cases (in all of which a defective intake of nicotinic acid may reasonably be presumed to have occurred) the excretions, although certainly subnormal and lower than any of those seen in the normal series, were yet *not vastly* below the normal range and in no single instance did they approach a zero value. It would be unprofitable to enter on any detailed discussion of this finding pending the completion of a more comprehensive survey of deficient cases and the establishment of standards of normality and deficiency, both for resting levels and for responses to test doses. In the meantime it is worth remarking that the conditions for the human may very well be found to resemble those already established in the above pages for the experimental animal: that is, that appreciable amounts of nicotinic acid may still continue to be excreted as the deficiency disease is developing, and therefore that it is not until a late stage of depletion has been reached that the excretion may be expected to fall to near zero. Advanced cases of pellagra are uncommon in this country and we have not yet had an opportunity of examining one. The alternative explanation would be that the test is not completely specific and that a residue of traces of other unidentified pyridine derivatives distinct from nicotinic acid are being measured in the deficient human subject by our test. The animal experiments lend no support to this view, however, since the ultimate value in the late avitaminosis is in fact zero.

Much laborious quantitative work will necessarily be required before standards of normality can be set up with any finality. The object of this paper is rather to describe the working details of the method recommended; and it can be claimed that the method in question does give reproducible results, that it permits of quantitative recovery of added nicotinic acid, that it differentiates between nicotinic acid and certain other pyridine derivatives (such as vitamin B₆ or trigonelline), that we have shown that the titre does in fact vary in accordance with the past intake and is responsive to test doses, and that deficiency in experimental animals can be satisfactorily diagnosed by its use.

SUMMARY

The process, which is based on the König colour reaction, is so planned as to circumvent the operation of various disturbing factors in urine referred to by Euler and by others.

The urine is heated with NaOH, to convert any amide into the acid, and neutralized. The specimen is divided into four portions; one is kept as blank, and to the other three are added 0, 20 and 40 μ g. of nicotinic acid. The solutions, which must be protected throughout from the light, are warmed with CNBr, cooled, treated with *p*-aminoacetophenone, allowed to stand and acidified, and the three colour intensities measured in a Pulfrich photometer with S 47 filter, comparing with the blank to which no CNBr has been added. For any given specimen the depth of colour varies with the pH, with the concentration of salts, and possibly with other factors, but the three readings always lie on a straight line and by extrapolation to zero the content of nicotinic acid can be accurately determined.

Duplicates agree well, within an error of about $\pm 10\%$, and added nicotinic acid or amide is quantitatively recovered.

In man the output varied according to the intake, and rose after test doses; lowered values were found in pellagra, and with anorexia. The normal range of values was usually from 3 to 5 mg. per day; but further detailed work is still needed to establish exact quantitative standards of normality.

Guinea-pigs or dogs deprived of nicotinic acid showed a progressive fall in the excretion as the symptoms of deficiency developed and, ultimately, a zero value. In rats the output was increased with a high intake of the vitamin, but the continued excretion on a deficient diet suggests some power of synthesis by the rat.

Vitamin B₆ does not interfere.

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Note added 13 December 1939. Following the preliminary publication of these results [Harris & Raymond, 1939] we have learned privately from Dr W. R. Aykroyd that very similar values for the excretion of nicotinic acid in urine have been found by Dr M. Swaminathan [paper in Press, *Indian Journal of Medical Research*].

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